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INVESTIGATIONS OF THE INFECTION BY
SCHISTOSOME PARASITES OF THEIR MOLLUSCAN
INTERMEDIATE HOSTS.

*A thesis submitted for the degree of
Ph.D. (Faculty of Science)
of the University of London.*

by

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April 1961

TABLE OF CONTENTS

	Page
Abstract	1
Acknowledgements	3
General Introduction	4
Historical Review	11
PART I	
PROTEINS AND AMINO ACIDS OF SNAIL BLOOD	
Introduction	15
Previous Work	16
A. Spectrophotometry of Snail Blood	
a) Method for obtaining blood	20
b) Preparation of blood derivatives	20
c) Method	21
d) Results	24
e) Comment	29
B. Electrophoresis of Snail Blood Proteins	
Introduction	33

	Page
a) Collection of blood	34
b) Preliminary experiments	34
c) Final technique	
Horizontal electrophoresis on cellulose acetate membrane strips	36
d) Experiments with the final technique	
Experiment I	
The effect of pH on the separation of blood proteins of snails	39
Experiment II	
Comparative rates of movement of haemoglobins from different snail species ..	42
Experiment III	
The occurrence of blood proteins other than haemoglobin in different species of snails	47
Experiment IV	
Electrophoresis of blood from snails maintained under different environmental conditions	58
Experiment V	
Electrophoresis of blood from <u>Lymnaea stagnalis</u> and <u>Oncomelania hupensis</u>	63

	Page
General Comment	66
C. The Analysis of Snail Blood Amino Acids by Paper Chromatography	
Introduction	70
1. Amino acids of snail blood pigments	
a) Preparation of samples	72
b) Preliminary experiments	75
c) Final technique	84
d) Results and Comment	
(i) Qualitative estimations of amino acids of snail blood proteins	103
(ii) Quantitative estimations of amino acids of snail blood proteins	107
2. Free amino acids of snail blood	
a) Preparation of samples	112
b) Method	114
c) Results and Comment	114
General Comment	118

PART II

PROTEIN AND NON-PROTEIN AMINO ACIDS OF SNAIL TISSUES OTHER THAN BLOOD

Introduction	120
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	Page
a) Preparation of samples	121
b) Results	124
c) Comment	126

PART III

AMINO ACIDS FROM TISSUES OF INFECTED AUSTRALORBIS GLABRATUS AND FROM CERCARIAE OF SCHISTOSOMA MANSONI

Introduction	129
a) Preparation of samples	130
b) Results	132
c) Comment	132

PART IV

SUMMARY AND CONCLUSIONS FUTURE WORK

Summary and Conclusions	136
Future Work	144
Appendices	146
References	151

INDEX OF TABLES AND FIGURES

	Page
TABLE I Absorption maxima for derivatives of snail haemoglobin 	25
FIGURES 1 - 3 Absorption spectra of snail haemoglobin derivatives 	26
FIGURE 1 Oxyhaemoglobin 	26
FIGURE 2 Carboxyhaemoglobin 	27
FIGURE 3 Cyanmethaemoglobin 	28
TABLE II Absorption maxima which occurred occasionally during spectrophoto- metric examinations 	30
FIGURE 4 Electrophoresis of snail blood proteins: The rate of movement of haemoglobin from different species	43
FIGURE 5 Electrophoresis of snail blood proteins: The separation of blood proteins in different species 	48
FIGURE 6 Separation of blood proteins in <u>A. glabratus</u>	49
FIGURE 7 Separation of blood proteins in <u>B(B). truncatus</u>	51
FIGURE 8 Separation of blood proteins in <u>B(P). nasutus</u> , <u>P. corneus</u> , <u>B(B). tropicus</u> and <u>A. nigricans</u> ..	53

	Page
FIGURE 9 Electrophoresis of blood proteins: Comparative rates of movement of serum proteins and snail blood pigments	64
FIGURE 10 Separation of blood proteins in <u>O. hupensis</u> , <u>L. stagnalis</u> and <u>A. glabratus</u>	65
TABLE III Chromatographic bands separated by the five solvents	86
TABLE IV Single amino acids separated by the five solvents	89
FIGURES 11 - 15 Separation of amino acids by circular paper chromatography using five solvent systems	92
FIGURE 11 Phenol pH 12.0	92
FIGURE 12 Phenol-butanol-acetic acid-water	94
FIGURE 13 Cresol pH 8.4	96
FIGURE 14 Butanol-acetic acid-water ..	98
FIGURE 15 Tertiary Amyl alcohol-benzyl alcohol-water	100
TABLE V Comparative amounts of selected amino acids in haemoglobin from three snail species	106
TABLE VI Free amino acids of snail blood	113

	Page
TABLE VII Free amino acids of the digestive gland and ovotestes 	123
TABLE VIII Free amino acids of snail anterior tissues 	125
TABLE IX Free amino acids from infected <u>A. glabratus</u> , and bound amino acids from <u>S. mansoni</u> cercariae ..	131

ABSTRACT

The respiratory pigments and the amino acid composition of planorbid snails which act as intermediate hosts for human schistosomes were compared with those of insusceptible snail species.

The absorption spectra of haemoglobins from different snail species were similar to one another and closely related to those of mammalian haemoglobins. The occasional appearance of additional absorption bands suggested that substances other than the pigment protein appear infrequently in snail blood.

Electrophoresis showed that, in general, the respiratory pigment was the only protein present in the blood. The mobility of haemoglobin and haemocyanin pigments was not affected by change in pH, and the rates of movement of haemoglobins from twelve different snail species were the same. Other proteins occurred occasionally in the blood of most species examined, and these additional fractions seemed to form a common pattern in four species. The factors determining their appearance were investigated.

No qualitative differences were observed in the amino acid content of haemoglobins from different snails, but quantitative differences were demonstrated.

The free amino acids of snail blood were in low concentration but were qualitatively similar in three snail species, which also contained similar bound and free amino acids in the digestive glands and ovotestes. Australorbis glabratus, however, differed from Planorbarius corneus and Lymnaea stagnalis since free methionine was found in its anterior tissues. The methionine was not present in glabratus snails infected with Schistosoma mansoni, and they contained lower concentrations of free amino acids than uninfected snails. It was shown that methionine was one of the seventeen amino acids identified in the protein of S. mansoni cercariae.

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GENERAL INTRODUCTION

The infection of snail intermediate hosts by schistosome parasites has been studied principally with regard to the degree of susceptibility of different species and strains of snails to various strains of the parasite. The results of many of these investigations have suggested the physiological differences exist between snail hosts of different species but, up to the present, few studies have been made on the comparative physiology of these intermediate and related non-intermediate hosts.

There are pronounced differences in the relative susceptibility of snail hosts to strains of schistosome species. Stunkard (1946), Cram, Files and Jones (1947) and Abdel-Malek (1950) were unable to infect the Egyptian intermediate host Biomphalaria boissyi with miracidia from a Puerto Rican strain of Schistosoma mansonii. Files and Cram (1949) also failed to infect B. boissyi with Venezuelan or Puerto Rican-Brazilian hybrid strains of the parasite. Files (1951) obtained moderate to high infection rates in Australorbis glabratus from Puerto Rico with strains of S. mansonii

from Puerto Rico, Surinam and Venezuela but very low rates with Egyptian and Brazilian strains.

On the other hand, successful infection rates have been obtained between snails and parasites from widely separated endemic areas. Kuntz (1952) infected strains of A. glabratus from Venezuela and Puerto Rico with an Egyptian strain of S. mansoni, the infection rates falling within the range recorded for the natural host B. Boissyi. Standen (1952) reported an infection rate of 82% in A. glabratus from Puerto Rico with S. mansoni from Egypt.

Considerable differences in susceptibility to infection of snail species from similar or adjacent localities have been reported. Barbosa (1958) reviewed the intermediate hosts of S. mansoni in Brazil. In some areas Tropicorbis centimetralis is the only snail which transmits schistosomiasis but it is a poor host; the majority of miracidia are walled off by a cellular reaction of the snail. A small number survive and develop but the life of the mature sporocyst is short and few cercariae are produced. Brooks (1953) showed a similar reaction in Tropicorbis havanensis and he suggested that successful development of the parasite

was due more to factors related to the miracidium than to invasion of a snail which was particularly susceptible. Barbosa showed that A. glabratus is an efficient intermediate host but A. nigricans is a poor host. Coelho (1957) reported, however, that the A. nigricans from Rio de Janeiro was completely refractory to infection by S. mansoni from Belo Horizonte and he considered that the high resistance to infection depended on factors related to the physiology of that molluscan species, rather than on a geographic differentiation of the parasite.

These results suggest that differences occur in the physiology of different strains or species of the intermediate hosts. A miracidium which develops readily in one intermediate host cannot always adapt itself to the conditions provided by another potential host. The survival or destruction of a miracidium after entering a snail depends upon the harmonious or incompatible relationship of the parasite and host. Successful development represents the physiological capability of the parasite to develop in the snail tissues and the physiological tolerance of the mollusc for the parasite (Kuntz 1952). The failure of a parasite to survive might represent the inability of the parasite

to utilise the physiological conditions in the snail rather than a direct immune response by the mollusc to the presence of the parasite. If a snail reacted directly to invasion by one strain of a parasite, it would probably react in a similar way to other strains. Similarly, where healthy sporocysts develop alongside miracidia which have been walled off by the intermediate host, the inference seems to be that a few miracidia have succeeded in adapting themselves to the snail whereas the majority have died and been walled off. The cellular phagocytic reaction of the snail seems, therefore, to be a secondary response to dead or dying miracidia.

Newton (1952, '53, '54, '55) has shown that genetic factors are involved in the susceptibility of two strains of A. glabratus to infection with S. mansoni. He crossed a highly susceptible pigmented Puerto Rican strain of A. glabratus with a refractory albino Brazilian strain and obtained susceptible snails from the progeny of the Brazilian parents. In snails of the second filial generation, tissue reactions characteristic of each of the parent populations occurred, namely no visible cellular reaction to the parasite by the susceptible stock and considerable cellular reaction by the refractory stock, with reactions intermediate between

the two. Selected breeding of the progeny produced a highly susceptible albino strain (Newton, 1955). He concluded that the Puerto Rican and Brazilian snails were genetically isolated with regard to infection, and variation in both the susceptibility of the snail and in the ability of the parasite to survive and develop occurred. Demonstration of variation in the parasite was difficult and Newton could not detect it in the reaction of miracidia in highly susceptible or highly refractory snails. When intermediates, of lower susceptibility, were obtained, a more variable set of conditions was available to the parasite and the fate of the parasites suggested variation in the miracidia; Newton could not determine whether this variability was due to genetic factors or to the condition of the miracidia after penetration.

Newton (1953) was able to infect the Brazilian strain of A. glabratus if very young snails, 2 - 16 days old, were used. The death rate was very high but the rate of infection and the death rate decreased with increasing age, no positives being obtained with snails over 5 weeks old (4-5 mm. in diameter). The young snails which became infected retained the infection long after the age at which they were normally refractory.

The physiological conditions in a snail will be genetically fixed, largely in response to its environmental conditions; different external conditions will probably affect the physiological state of a snail species in different ways until strains of the species that are not reproductively isolated may become distinct from one another with regard to those aspects of the internal physiology which are influenced by the external environment. In a similar way, different physiological strains of a parasite species may be expected to occur, since a successful parasite must be adapted, to a considerable extent, to the environment provided by its host. As the adaptation of the parasite to its host becomes more specialised, the parasite becomes more host restricted and less able to develop successfully in a different set of conditions.

Infection of snail intermediate hosts by schistosomes has indicated probable physiological differences between different snail species. The principal American and African schistosomes are confined to Planorbidae snails. S. haematobium and S. mansoni are restricted to the Bulininae and Planorbinae respectively. Only one instance is recorded of a Planorbinae snail, P. dufouri, acting as the intermediate host of S. haematobium (Bettencourt and Borges 1922). In addition, strains of each species have

been shown to vary greatly in their ability to develop in different potential hosts. It may be noted here that miracidia from an Egyptian strain of S. mansoni will penetrate the British planorbid Planorbarius corneus (Targett, unpublished). Sporocysts were found in these snails eighteen hours after exposure but further observations on the extent of development of the parasite have not yet been made. This indicates, without considering the evidence of chemical attraction by the snail, that factors governing the susceptibility to infection can be effective after the parasite has penetrated a potential host.

Comparative physiological studies of known and potential intermediate hosts may show what variation occurs between snail species and thus what conditions are available to the parasite. These studies may further indicate factors which limit the distribution of different strains and species of schistosomes. Three investigations into comparative molluscan physiology are reported in this thesis.

HISTORICAL REVIEW

There are some interesting comparative studies on the physiology of intermediate hosts of trematode parasites. Olivier et al (1953) found a quantitative difference in anaerobic carbohydrate metabolism between Dominican and Puerto Rican strains of Australorbis glabratus. The level of anaerobic metabolism of both infected and uninfected Dominican snails was higher than that of the Puerto Rican strain.

The difference in susceptibility to infection with a S. mansoni strain of a Brazilian and a Venezuelan strain of A. glabratus has already been mentioned (Files and Cram 1949). Newton and Haskins (1953) compared the susceptibility of these snails, and Brazilian albino mutant and Puerto Rican strains, to sodium pentachlorophenate. The Brazilian albino mutant strain was most susceptible and the Brazilian, Venezuelan and Puerto Rican snails increasingly more resistant. Newton and von Brand (1955) considered that these differences suggested physiological differences between the strains and they compared the Brazilian and Venezuelan snails with regard to chemical composition, polysaccharide formation from selected carbohydrate diets, aerobic and anaerobic respiration, and anaerobic survival. They found no

significant differences in the inorganic substances, ether soluble fractions and the nitrogen content of the two strains. With snails of a similar weight range the shells of the Venezuelan strain were heavier than those of the Brazilian one. The Venezuelan stock stored about twice as much polysaccharide as the Brazilian strain, and, by feeding the snails on selected food materials, they demonstrated an equal synthesis of polysaccharide in the two strains, showing that the difference in polysaccharide levels of snails maintained on normal diet was not due to differences in methods of synthesising or storing capacity. Both strains had an identical rate of oxygen consumption but the Brazilian strain produced less carbon dioxide and survived for much shorter periods under anaerobic conditions. This was found to be due to the lower level of stored polysaccharide. Newton and von Brand considered that these differences were genetically fixed by geographical separation of the two strains.

Olivier (1956) examined strains of A. glabratus and T. centimetalis to determine their ability to survive out of water in the laboratory. Within each species, snails from different habitats varied in the length of time they were able to survive desiccation, and Olivier suggested that these variations were due, in part, to

physiological differences between the strains.

Variations in the chemical composition of mucus of snails have been demonstrated (Wright 1959, Wright and Claugher 1959). Chromatographic analyses of mucus from different snail species revealed substances of unknown chemical nature, which fluoresce in ultra-violet light, and the patterns produced showed relationships and differences between species of Lymnaea. Differences were also found in the patterns of mucus obtained from species of Bulinus. Wright (1960) described variations in patterns obtained from races of Lymnaea peregra and stated that these variations occur most frequently near the edge of a species range. He further suggested that new trematode-snail relationships are likely to occur in this region, assuming that variations in other characteristics are also most likely on the fringe of a species range.

Von Brand and Files (1947) investigated the effect of infection with S. mansoni on the physiology of A. glabratus. Infection produced no significant change in the fat content or oxygen consumption of these snails but the polysaccharide content decreased. Histochemical studies showed that infected snails contained less glycogen in the muscles than uninfected ones. In some specimens the tubules of the digestive gland had lost all their

glycogen, but in others the glycogen had disappeared from the epithelial tissues. Reduction of glycogen was also observed in the ovotestes, due primarily to the fact that infected snails contained fewer developing eggs. The amount of galactogen in the albumen gland did not appear to be significantly altered.

Sporocysts had a low glycogen content but the amounts present in cercariae were variable. In mature cercariae the greatest amount of polysaccharide was found in the tail stem. Von Brand and Files were unable to determine the factors which produced the decrease in polysaccharide content but they suggested that it was due either to an impaired carbohydrate digestion, to toxic action by the parasite, or to deprivation of the snail by the parasite of essential nutrients.

PART I

PROTEINS AND AMINO ACIDS OF
SNAIL BLOOD

Proteins and Amino Acids of Snail Blood

Introduction

The blood system of planorbids has very few vessels, the snail tissues being bathed directly by the circulating blood. Schistosome sporocysts are thus exposed to the blood during part, at least, of their development. A comparative study of the composition of blood of different snail species seemed a logical preliminary to investigation of the physiology of intermediate and non-intermediate hosts. Abdel-Malek (1955) found that schistosome miracidia penetrated blood spaces and connective tissue beneath the epiderm and developed into mother sporocysts in these regions. The daughter sporocysts liberated from the mother sac were carried by the blood, the majority settling in spaces between the vascularised tubules of the digestive gland and acini of the ovotestes to continue their development to maturity.

Proteins contained in snail blood are known to function as buffers (Prosser et al 1952), their effectiveness being due to the fact that each protein molecule may have several acid-binding groups. A difference in the blood proteins could, therefore, produce different degrees of buffer action and in consequence a variation in the inorganic constituents of the blood.

The pigment protein also provides blood colloid (Redfield 1934). The level of blood protein, therefore, helps to maintain osmotic equilibrium with the environment, and the blood system, in its role as a general medium whose constituents are important in the maintenance of the stable internal environment of the mollusc, must play some part in determining the external environment of the developing schistosome parasite.

The blood proteins were also examined to determine their possible taxonomic value. There is a need for clarification of the taxonomy of intermediate hosts of schistosomes, and examination of blood from species distinct from one another might indicate whether blood characters could be of value in settling some of the many problems of classification.

Previous Work

Baker (1945) and Pan (1958) have described in detail the circulatory system of planorbid snails. It consists of a heart, arterial and venous systems and blood sinuses. The arteries branch freely and Pan (1958) considers that blood is forced into loose vascular connective tissue. After bathing the tissues the blood collects in blood sinuses which have no limiting wall and these merge with veins which also have no definite wall.

There are no blood cells as such and the pigment, haemoglobin or erythrocrucorin⁺, is dissolved in the plasma.

Nucleated amoebocytes, which appear to function as phagocytes, have, however, been observed in small numbers in the circulatory and connective tissues.

The haemoglobin, like all blood pigments which are dissolved in plasma, has a high molecular weight. Svedberg (1933) suggested that large molecules such as these were composed of many units, each of molecular weight approximately 34,500. Svedberg and Pedersen (1940), obtained an isoelectric point of 4.77 for the haemoglobin of Planorbis corneus (syn. Planorbarius corneus) and calculated, from the sedimentation constant obtained with an ultra-centrifuge, that the molecular weight was 1,539,000.

Wright and Ross (1959) examined the proteins of the blood of Australorbis glabratus by horizontal electrophoresis on cellulose acetate membrane strips using 0.02M barbitone buffer solutions as electrolytes. In most

⁺ Keilin and Hartree (1951) compared the physico-chemical properties of vertebrate and invertebrate blood pigments and showed that the distinction made between haemoglobin and erythrocrucorin was not valid. The snail blood pigment will therefore be referred to as haemoglobin.

samples the only protein band visible was haemoglobin, but with snails 7-10mm. diameter they obtained separation of other bands, the best separation occurring at pH 11.6.

Sorby (1876) compared the positions of spectral absorption bands for vertebrate haemoglobin and oxygenated haemoglobin from Planorbis. He obtained maxima for vertebrate haemoglobin at 581 mu. and 545 mu., and at 578 mu. and 542.5 mu. with snail blood. He stated that, even if these figures were not absolutely correct, the absorption bands from Planorbis haemoglobin were clearly more near the blue end of the spectrum. Anson et al (1924) examined blood from Planorbis corneus with a spectroscope and found that the absorption maxima for oxyhaemoglobin was 574.6 mu. and the maximum for carboxyhaemoglobin was 570.8 mu.

Only isolated data are available of the functions of the blood haemoglobin by direct studies on snails, the functions of respiratory pigments being largely deduced from results obtained with snails containing haemocyanin. Leitch (1916), Fox (1945) and Prosser et al (1952, review) showed that haemoglobin in Planorbis appeared to function in oxygen transport primarily at times of physiological stress due to hypoxia. Oxygen carried in solution was normally sufficient for the animal down to a water tension

of 7.7% oxygen, and Leitch estimated that the oxygen held by the pigment would last three minutes during anoxia. Borden (1931), however, estimated from data of the oxygen capacity, volume and oxygen consumption of the blood, that oxygen held by the haemoglobin would last eighteen minutes during anoxia.

A. Spectrophotometry of Snail Blood

a) Method for obtaining blood

The snails were dried thoroughly by blotting with filter paper. The shells of flat species were pierced on the inside of the innermost whorl and, if the blood did not run freely, the snail was eased back in the shell with a small piece of filter paper. This was done carefully as the snails bleed easily through the foot and the blood becomes contaminated with mucus. Blood was obtained from snails with a spire by piercing the tip of the spire, the snail being eased back into the shell in a similar way, if necessary. Small specimens of each type were pierced and bled on the stage of a low-power dissecting microscope. The blood was collected with a fine Pasteur pipette. Snail blood does not clot and the colour varies according to the amount of haemoglobin present, which in turn depends on the conditions under which the snails have been maintained. It is usually bright red but the colour deepens when snails are kept in water of low oxygen concentration.

b) Preparation of blood derivatives

(1) Oxyhaemoglobin

Undiluted blood was exposed to air for at least one hour.

(2) Carboxyhaemoglobin

Coal gas was bubbled through undiluted blood for one hour.

(3) Cyanmethaemoglobin

Blood was diluted with several volumes of a reagent prepared by adding 1.0g. sodium bicarbonate, 50 mg. potassium cyanide and 200 mg. of potassium ferricyanide to one litre of distilled water. (Wootton, personal communication).

Subsequent dilutions of all derivatives were made with $\frac{N}{150}$ ammonia solution.

(c) Method

Preliminary readings were made on a Unicam S.P. 600 spectrophotometer using 2 mm. cuvettes. The majority of results were, however, obtained with a Unicam S.P. 500 spectrophotometer fitted with a hydrogen discharge lamp, using 1 cm. cuvettes.

Readings were made over the wavelength range 220-600 mμ. at intervals of 5 or 10 mμ., except in regions of peak absorption when the smallest intervals that were convenient, 0.5 or 1.0 mμ., were used. For the longer wavelengths blood was diluted 4 or 10 times and diluted further for shorter wavelengths, up to 500 times being

necessary in the ultra-violet range. The dilutions were adjusted to keep the readings within the optimum recording range of the instrument, 20-80% transmission. The blank solution was $\frac{N}{150}$ ammonia solution.

Results were recorded as optical density and, from these, extinction coefficients were calculated as follows:

$$T = 10^{-ecd}$$

T = transmission

$$d = -\log T$$

d = optical density

$$d = ecl$$

e = extinction coefficient

$$e = \frac{d}{cl}$$

c = concentration

l = width of cuvette

The concentration of haemoglobin was expressed as milligrammes of iron per cent. This standard was used to ensure, as far as possible, that the concentration referred only to the haemoglobin since the iron-containing haem portion of this molecule produces the important absorption peaks.

Iron determinations were made using the method devised by Wootton (1958) for estimation of iron in biological material by spectrophotometry of ferric perchlorate. A sample was heated on a hotplate with AnalaR nitric acid until a brown or yellow mobile liquid resulted. AnalaR perchloric acid (10N) was added to

the cooled solution and the mixture evaporated to dryness over $1\frac{1}{2}$ - 2 hours. The residue was dissolved in 10N perchloric acid by boiling gently and, after cooling, water was added to make the final solution N. perchloric acid. A permanent standard was prepared by dissolving 500 mg. of pure iron in 60 ml. of AnalaR nitric acid and 100 ml. of water and diluting to one litre. A volume of this stock standard was evaporated to dryness with 10N perchloric acid. The residue was dissolved in 10N perchloric acid and diluted to a normal solution with water.

The blank was N. perchloric acid and readings were made on a Unicam S.P. 500 quartz spectrophotometer at 240 mu. using 1 cm. silica cuvettes. The optical density was found to be proportional to the iron concentration up to a density of 0.8.

Rather than rely on Wootton's single standard, a range of standards was prepared containing 0.2 - 1.0 mg. of iron/100 ml., to give a true calibration curve. Samples of 0.1 - 0.4 ml. of blood were used for estimation of iron in snail haemoglobin, to give a final volume of 10 ml. of ferric perchlorate.

Derivatives of haemoglobin from four species,

Australorbis glabratus, Planorbarius corneus, Bulinus
(Bulinus) tropicus angolensis and Bulinus (Physopsis)
globosus, were prepared.

(d) Results

The wavelengths of maximum light absorption for derivatives of the four species of snails are given in Table 1, with results for two derivatives of mammalian haemoglobin (Lemberg and Legge 1949) included for comparison. Figures 1-3 show absorption spectra for the three derivatives of haemoglobin from A. glabratus and P. corneus (Targett 1959). The absorption peaks obtained in the visible region of the spectrum (>400 m μ .) for each species were constant within 1 m μ ., the limit of accuracy of the spectrophotometer in this region. A single peak for the carboxyhaemoglobin derivative of B(B) tropicus angolensis showed a greater range for the position of the wavelength of maximum absorption, and this is recorded in the Table. In the ultra-violet region the positions of the individual maxima were slightly more variable and the peaks recorded for each derivative in Table I are those which occurred most frequently. The patterns of haemoglobin maxima shown in Table I occurred regularly during many examinations of different samples

TABLE I

Absorption Maxima for Derivatives of Snail Haemoglobin

<u>Oxyhaemoglobin</u>	<u>Absorption Maxima mμ</u>				
A. glabratus	276	348	414	501	540 574
P. corneus	276	347	414	502	539 574
B(B) tropicus angolensis	276	347	413	-	539 575
B(P) globosus	279	346	414	-	539 575
Mammalian blood [†]	270-80	330-40	412-15	-	540-42 576-78

Carboxyhaemoglobin

A. glabratus	275	348	419	502	539 567
P. corneus	279	346	420	502	539 567
B(B) tropicus angolensis	279	346	420	502	539 567-9
Mammalian blood [†]	270-80	330-40	418	-	538-40 568-72

Cyanmethaemoglobin

A. glabratus	274	348	419	500	537 -
P. corneus	279	-	419	500	537 -
B(P) globosus	275	-	419	-	537 -

[†]Lemberg and Legge (1949)

FIGURE 1

Absorption Spectra of Snail Haemoglobin Derivatives

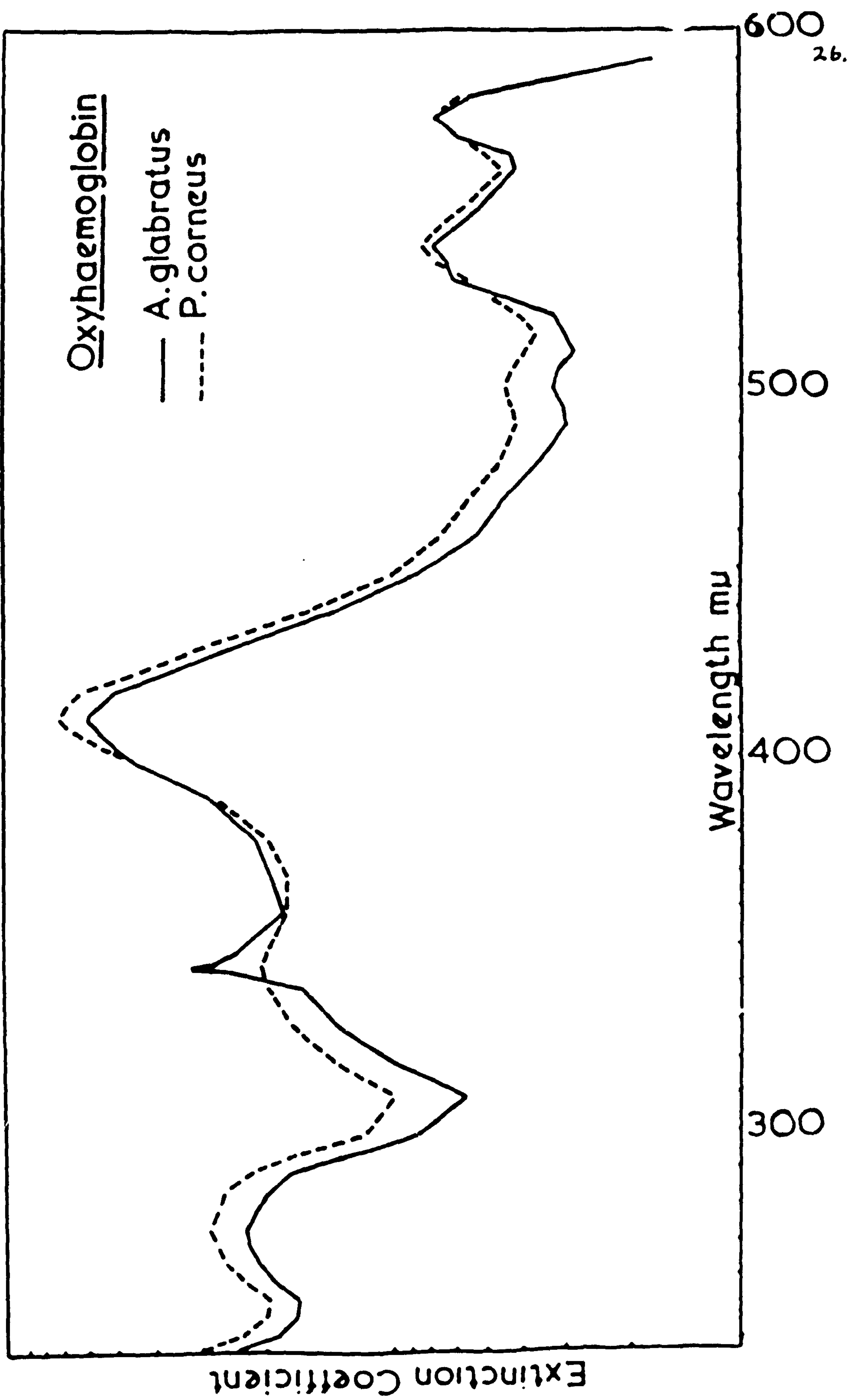


FIGURE 2

Absorption Spectra of Snail Haemoglobin Derivatives

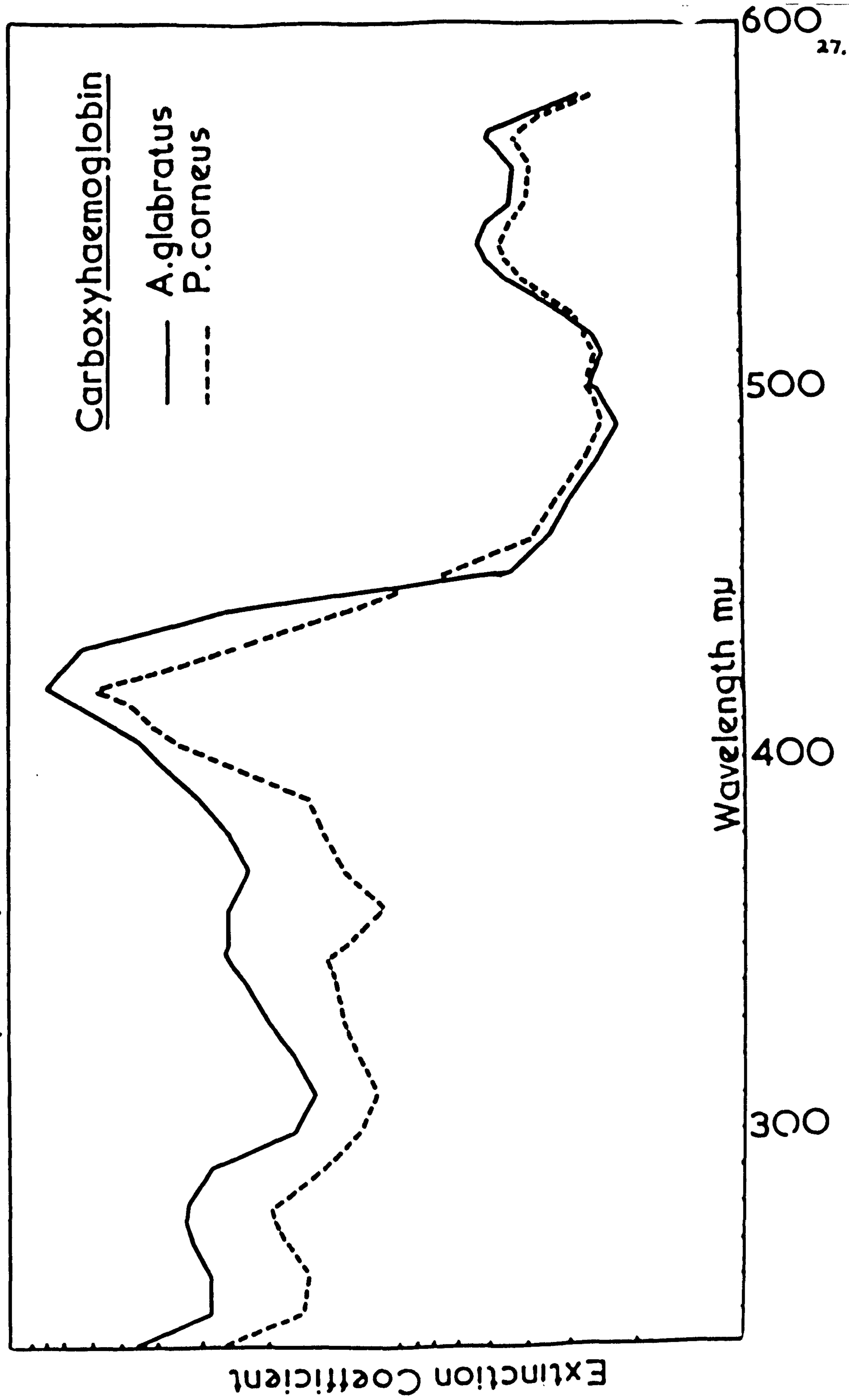
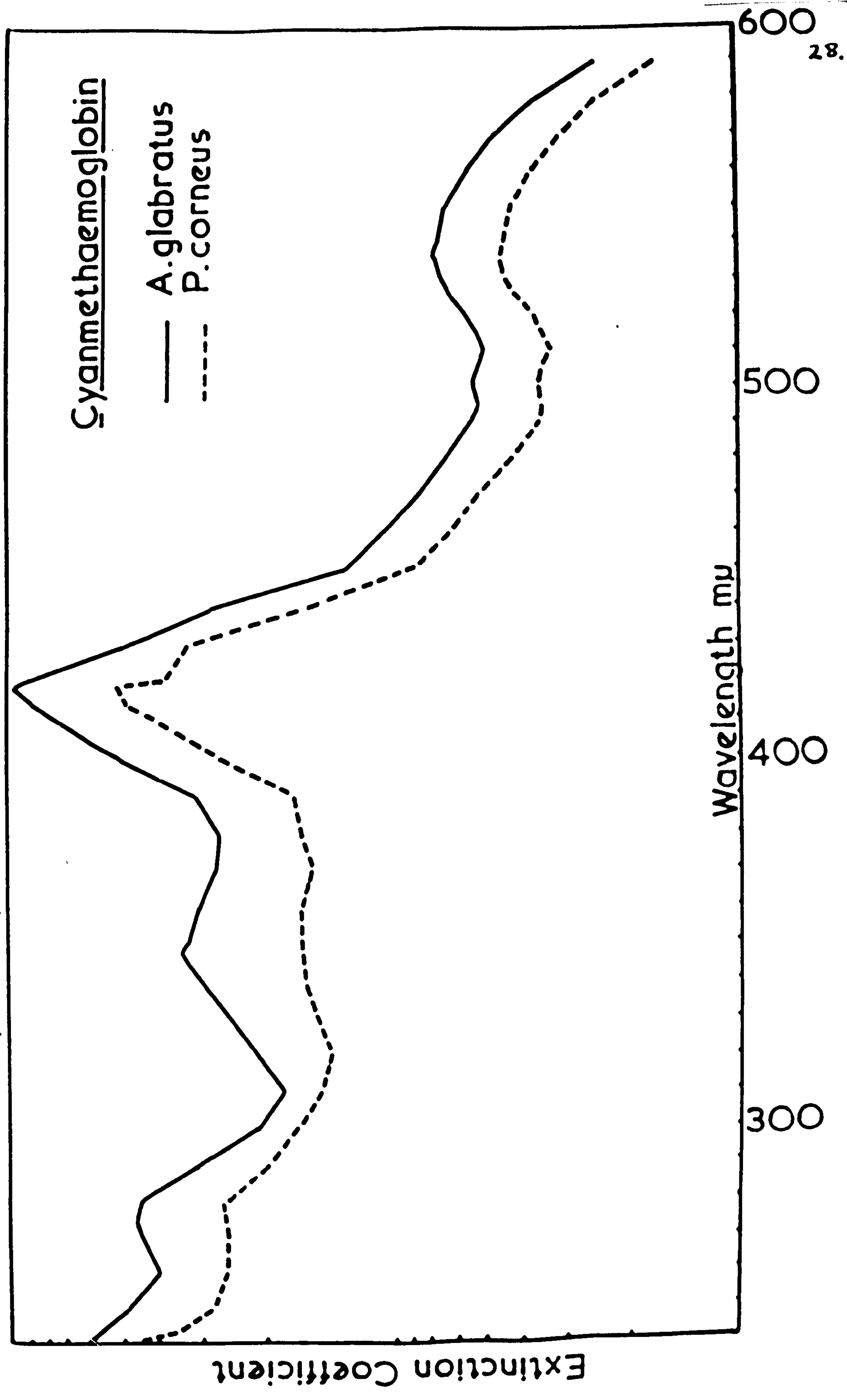


FIGURE 3

Absorption Spectra of Snail Haemoglobin Derivatives



of blood, but, in addition, other small absorption peaks were present in the ultra-violet in each species (Table II). However, since these occurred only infrequently they were not considered typical of the absorption spectra of the haemoglobins.

(e) Comment

The spectrum of haemoglobins depends on the derivative examined and the structure of the prosthetic group and protein portion of the molecule (Lemberg and Legge, 1949). Variations in the prosthetic groups of the molecule produce more marked spectrophotometric differences than differences in the protein portions since most organic substances absorb light at wavelengths below 250 mμ; the absorption of light of longer wavelengths is usually associated with the presence in the molecule of unsaturated linkages (Fruton and Simmonds, 1956). Porphyrins, which form the prosthetic groups of mammalian haemoglobins, have a characteristic form of spectrum in the visible region (400 - 650 mμ) although the absorption maxima may vary with different porphyrin groups.

The results indicate the similarity in positions of the maxima for invertebrate and vertebrate haemoglobins, and the form of the spectrum for snail haemoglobins,

TABLE II

Absorption maxima which occurred occasionally
during spectrophotometric examinations

<u>Oxyhaemoglobin</u>	<u>mμ</u>					
A. glabratus	-	282				
B(P) globosus	265	-				
<u>Carboxyhaemoglobin</u>						
A. glabratus	-	-	-	-	358	365
<u>Cyanmethaemoglobin</u>						
A. glabratus	-	-	-	-	358	-
P. corneus	265	-	-	-	-	370
P. globosus	264	-	302	324	359	-

Figures 1-3, is very similar to that obtained with mammalian haemoglobins (Lemberg and Legge 1949) showing a high Soret band (412-20 mμ). Oxy- and carboxyhaemoglobins also show the two bands characteristic of mammalian haemoglobin in the ultra-violet region, although the maxima at the longer wavelength are significantly higher in the snail species (346-348 mμ) than in mammalian blood (330-340 mμ).

Absorption bands in the ultra-violet, below 400 mμ, may be modified by the protein portion of the molecule (Lemberg and Legge 1949). The different snail species showed some apparent differences in the position of maxima in this region (Table I), but the wavelengths of maximum absorption obtained with different samples of blood from one species also varied, and the results obtained for the different species overlapped, indicating that these variations were not significant.

Comparison with mammalian haemoglobins indicates that the small absorption band at 500-502 mμ was due to a small amount of methaemoglobin (Lemberg and Legge 1949). This peak was never obtained with blood from B(P) globosus.

The occasional appearance of other absorption bands in the ultra-violet region (Table II) suggests that substances other than haemoglobin occur sporadically in the

blood. Amino acids generally absorb light below 250 m μ . although phenylalanine, tyrosine and tryptophane show absorption bands at 260, 270 and 280 m μ . respectively, and the presence of these substances would produce peaks in this region (Fruton and Simmonds 1956). Since the protein portion of the haemoglobin molecule can modify the absorption spectrum below 400 m μ . of prosthetic groups, these additional bands, which always produced only small peaks, may have been due to proteins, other than haemoglobin, in the plasma.

The results show the close similarity between the spectra of the different snail species and mammalian haemoglobin. Since the spectral pattern is produced largely by the prosthetic group of the molecule, it is clear that the porphyrins of the snail haemoglobin are qualitatively similar to one another and to the porphyrin groups of mammalian blood.

B. Electrophoresis of Snail Blood Proteins

Introduction

Early investigations of snail blood were concerned with the properties of the blood pigment and did not consider the possibility of other proteins in the blood. Wright and Ross (1959) showed, however, that under certain conditions, proteins other than haemoglobin could occur in blood of A. glabratus.

Electrophoresis, under varying conditions, of blood from snails would give quantitative comparison of the proteins present in the blood. In addition, it would indicate whether there were any differences between the haemoglobin pigments of different snails. The rate of movement of a protein in an electrical field depends, in part, on its electrical mobility which in turn is governed by the number and type of ionisable groups in the amino acid side-chains. These groups are also important physiologically since they can bind inorganic ions (Fruton and Simmonds 1956) and the mobilities of the haemoglobins might indicate whether any differences in the ion-binding properties of the molecules occurred. The examinations would further indicate whether blood protein characters could be of use in taxonomic studies of the snails.

a) Collection of blood

Blood samples were obtained by the method previously described for the spectrophotometric analyses, except that the collection was made in finely drawn capillary tubes. Since only very small quantities were required it was not necessary to kill the snails and, frequently, samples of blood were taken from a single snail over a period of many weeks.

b) Preliminary experiments

(1) Hanging strip paper electrophoresis

Methods

0.025 ml. samples of human serum and blood from A. glabratus and P. corneus were applied separately to 5 cm. wide strips of Whatman 3 MM. paper. The strips were irrigated in standard vertical type electrophoresis tanks (see Block et al 1958) for 6-8 hours at 5 milliamps/strip. Two series of experiments were performed, one with 0.05M barbitone buffer at pH 8.6, the other with 0.1M acetate buffer at pH 5.2 (Appendix II). The strips were stained with Azocarmine B (Appendix I).

Results

At pH 5.2 the snail haemoglobins barely moved from the point of application and formed rather irregular bands. At pH 8.6 some movement occurred and a single dense spot,

which tailed to the origin, appeared. When compared with human serum, in which the α_2 globulin band remained at the point of application, the snail haemoglobins had a mobility similar to α_1 globulin.

(2) Horizontal paper electrophoresis

Apparatus

This consisted of a small electrophoresis tank with separate buffer and electrode compartments and a bridge holding a 5 cm. width paper. Current was supplied from a constant current power-pack. The buffer was 0.05M barbitone solution pH 8.6 (Appendix II) and the strips were stained with 1% light green in 1% acetic acid, Azocarmine B or 1% nigrosine (Appendix I).

Method

The buffer chambers were filled to approximately the same level and the tank equilibrated for one hour with a strip of filter paper on the bridge. Strips of Whatman 3 MM paper, 2.5 X 20 cm., were soaked in buffer solution and samples applied either with the strip in position on the bridge or to blotted strips before they were placed in the tank. The point of application was varied from 0 - 4 cm. from the centre on the cathode side. 0.005 ml. samples of baboon serum and snail blood were applied from

graduated pipettes. The strips were irrigated for 3-4 hours at 0.5-1.0 ma/cm.

Results

Application of samples 2.5 cm. from the centre with a current of 0.5 ma/cm. for 3.75 hours gave good separation of serum proteins but snail haemoglobin moved very little. The greatest movement of snail haemoglobin occurred with an application point 4 cm. from the centre, with a current of 1 ma/cm for 3 hours. The haemoglobin formed a strong spot 2-3 cm. from the point of application but there was considerable tailing.

These results suggested that electrophoresis on paper was unsuitable for separation of snail blood proteins.

c) Final technique - Horizontal electrophoresis on cellulose acetate membrane strips.

Apparatus

The electrophoresis tank developed by Kohn (1957 and 1958) as a micro-technique for separation of serum proteins consists of two outer buffer chambers each connected by wicks to one of two inner electrode chambers. The four compartments are filled with buffer solution. The cellulose acetate strips rest on an adjustable bridge and are tensioned by strip holders which are lined with filter paper, the filter paper making electrical connection

through buffer solution in the buffer chamber.

Current (D.C.) was supplied from a constant current power pack supplying up to 15 ma.

Reagents

Various buffer solutions were used (Appendix II). Strips were stained with 0.005% nigrosine (Appendix I).

Method

The buffer and electrode compartments were filled to approximately the same level with buffer solution. Strips of filter paper soaked in the buffer were placed in position on the bridge and the tank equilibrated for at least two hours. Cellulose acetate strips, 2.5 x 10 cm., were floated on the buffer solution, lightly blotted and placed in position on the bridge. Up to eight strips could be run at one time. Samples were applied as thin streaks, 1.0-1.5 cm. long, from graduated pipettes or finely drawn capillary tubes in amounts of 0.001-0.005 ml. Current was passed for the required time and the strips were dried for 10-30 minutes at 70-80°C, stained with 0.005% nigrosine, washed, and dried between sheets of blotting paper. The strips were cleared for scanning by immersion in Whitemer 120 oil (Manchester Oil Refinery Ltd.) and scanned with an Eel transmittance densitometer.

Considerable care was necessary when handling the membrane strips as they mark easily and finger marks show after staining.

The patterns obtained and the rate of movement of the proteins were greatly influenced by the buffer levels, the ionic strength of the buffer, the amount of current and the length of the experiment. The pH of the buffer was an important factor in separation of the snail proteins.

The distance between the membrane strip and the level of the buffer affected the rate of movement of proteins. Movement was slower with low buffer levels and the bands were more narrow. Buffers of lower ionic strength produced faster movement but more diffuse separations, and the level of the buffer was adjusted according to its ionic strength. In general, a buffer level 2-3 cm. below the membrane strip was most satisfactory. Increase in current and the length of run produced faster movement, but a current of 0.3-0.4 ma/cm. for 2-3 hours generally produced satisfactory results.

Initially, the point of application was varied in order to find the optimum position. Application 3 cm. from the centre on the cathode side gave good results and this was used as the standard position in the majority of experiments.

d) Experiments with the final technique

Experiment I:

The Effect of pH on the Separation of Blood Proteins of Snails

Wright and Ross (1959) ran blood of A. glabratus with 0.02M barbitone buffers at different hydrogen-ion concentrations and obtained best separations at pH 11.6. The different pH levels were obtained by adding sodium hydroxide solution to soluble barbitone (Ross, personal communication). Similar experiments were therefore performed with blood from A. glabratus and Planorbarius corneus using different buffer solutions of pH 8.6-12.0. The snails varied in size from 3-17 mm.

Results

A. glabratus generally showed a similar pattern with most of the buffer solutions used. This was a single protein band, the haemoglobin, which tailed slightly to the point of application. The rate of movement of the haemoglobin varied according to the conditions employed with each buffer but, by altering the level of buffer and amount of current for each solution, it was possible to obtain, with all but one of the solutions used, a fairly compact band. This was never obtained with the barbitone-NaOH solution pH 11.6 of low ionic concentration (Appendix IIc No. 6), the haemoglobin forming either a

very diffuse broad band or simply streaking irregularly from the point of application. This occurred also with solutions of lower pH if the samples were applied close to the centre of the strip (0.2 cm. from the cathode), or if the level of buffer in the tank was too high. The best bands were obtained with very alkaline solutions of pH 11.2-12.0 (Appendix IIc, nos. 2, 5, 7), haemoglobin forming a more compact band than at lower pH levels, with less tailing. The most satisfactory results were finally obtained with the very alkaline solutions by adjusting the level of buffer and amount of current to give band movement of approximately 3 cm.

The pattern generally obtained was a single protein band but occasionally other proteins occurred, particularly with buffer solutions of pH 11.2-12.0. These usually form fairly distinct bands at the high pH levels although occasionally there was only a diffuse darkly stained area behind the haemoglobin and a lightly stained area ahead of it. At lower pH levels, no distinct separations of other protein bands were obtained, although samples occurred which showed diffuse stained areas, suggesting the presence in the blood of substances other than haemoglobin.

P. corneus was examined in the same way and the

behaviour of the haemoglobin was very similar to that of A. glabratus. Additional blood proteins were also obtained but on very few occasions. The separations were again shown most clearly with highly alkaline solutions, the solutions of lower pH producing the indeterminate stained areas.

Comment

The only protein normally present in the snail blood was the respiratory pigment. Separation of the haemoglobin, while not affected greatly by the pH of the buffer solution, was somewhat better at very high pH, with less tailing. This tailing was generally only slight and was found to be due to haemoglobin.

In a few snails blood proteins other than haemoglobin were found to occur with solutions of pH 11.2-12.0. These results will be considered later. The indeterminately stained areas which occurred at lower pH levels probably represented the same substances since a sample from A. glabratus which produced bands at pH 11.6 (0.05M barbitone - 0.2M NaOH buffer Appendix II), showed these diffuse bands at pH 8.6 with 0.05M barbitone buffer (Appendix II).

Experiment II

Comparative Rates of Movement of Haemoglobins from Different Snail Species by Electrophoresis with Buffers of High Alkalinity.

The rates of movement of haemoglobins from different snail species were compared using buffer solutions of pH 11.2-12.0 (Appendix II). The following species were examined:-

1. Australorbis glabratus (Puerto Rican strain, laboratory-bred for many years).
2. A. glabratus (El Torro, Puerto Rico).
3. A. nigricans
4. Biomphalaria sp. (Central Nyanza, Kenya).
(? B. Pfeifferi ruppellii).
5. Biomphalaria sp. (Mwilulu, Central Nyanza, Kenya)
(? B. sudanica tanganyicensis)
6. Tropicorbis centimetralis
7. Bulinus (Bulinus) truncatus (Cairo)
8. B.(B). truncatus (Wittenberg)
9. B.(B). tropicus angolensis
10. B. (Physopsis) globosus (Tanganyika)
11. B.(P). nasutus (Kenya)
12. Planorbarius corneus (Laboratory-bred for several years).

Results

Figure 4 shows the comparative rates of movement of

ELECTROPHORESIS OF SNAIL BLOOD PROTEINS

The Rate of Movement of Haemoglobin from Different Species

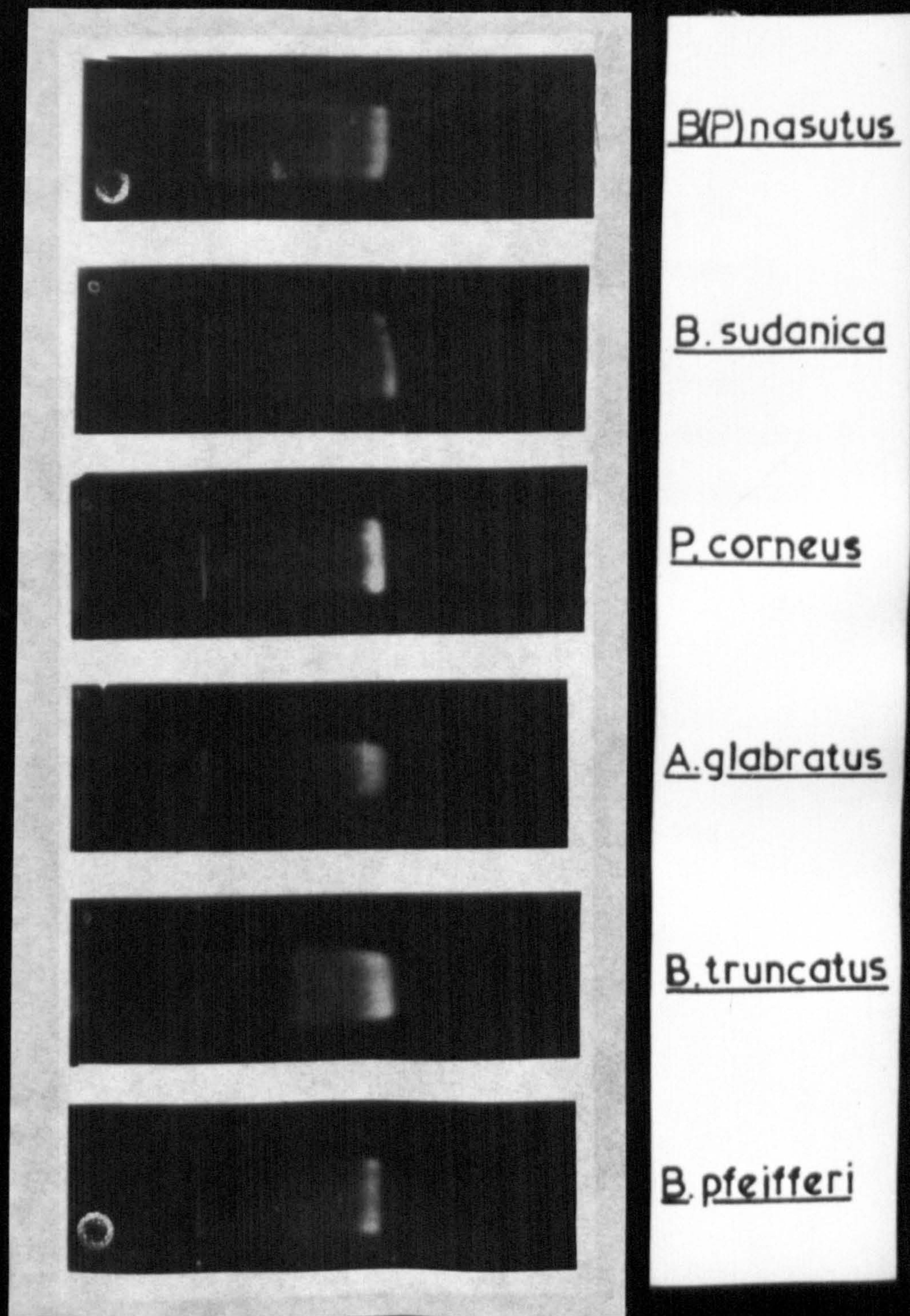


FIGURE 4

haemoglobins from six species. Many similar experiments were performed with numerous samples from each snail species. Slight variations in the experimental conditions sometimes affected the rate of movement of haemoglobin from a single species so that direct comparison of the mobilities of haemoglobins examined in different experiments had to be made with caution. A series of experiments were performed at pH 11.6 (Appendix IIc, No.2), under similar conditions with blood from the twelve different species. The results indicated that the blood pigments from all the snails examined showed very similar rates of movement, and the patterns obtained were similar to those described for A. glabratus (Experiment I). In general the blood contained only haemoglobin but occasionally other proteins were present and showed either as distinct bands or as diffuse stained areas. Figure 4 shows these lightly stained areas in two specimens, B.(B). truncatus and B.(P). nasutus. The rate of movement of the haemoglobin fraction was never altered by the presence in the blood of other proteins.

A few studies with buffer solutions of pH 8.6-11.0 indicated that the behaviour of the haemoglobin fractions of all the snails was similar to that of A. glabratus (Experiment I).

Wright and Ross (1959) found that the haemoglobin of A. glabratus had a mobility similar to that of human serum albumen. During the present investigation, blood from A. glabratus was run alongside human and baboon sera at pH 8.6 and 11.6. The amount of current and length of run were varied in different experiments but the snail haemoglobin always ran more slowly than the serum albumens (Fig. 9). The mobility of the pigment was similar to that of α_2 globulin. The relative rates of movement of serum albumen and snail haemoglobin remained similar under the various experimental conditions and with different buffers so that no reason can be suggested for the difference in our results.

Comment

The behaviour of blood from snails, when run under different experimental conditions, indicates that, in general, haemoglobin is the only protein present. This is significant with regard to its function as a buffer and blood colloid and will be discussed later.

The snail haemoglobins have the same mobilities under the conditions of these experiments, and from a taxonomic point of view, at least, the twelve species show no reproducible differences. Spectrophotometry of representative species of this group of snails has already

shown that the prosthetic groups of the molecules are the same and these results suggest that the form of the whole molecule is probably similar in the different species.

The occasional appearance of other blood proteins will be considered in more detail later and they are important here only for the effect they have on the blood pigment. The rate of movement of the haemoglobin band was not altered when the other fractions were present and the haemoglobin was always the principal component. Haemoglobin always showed slight tailing to the point of application. When other proteins were present there was rather more background colour but it was not possible to determine whether this was due to the haemoglobin. It was impossible to relate the presence in the blood of extra proteins to the quantity of haemoglobin since the latter varies greatly in different snails even when they are maintained under similar environmental conditions.

Experiment III

The Occurrence of Blood Proteins other than Haemoglobin in Different Species of Snails

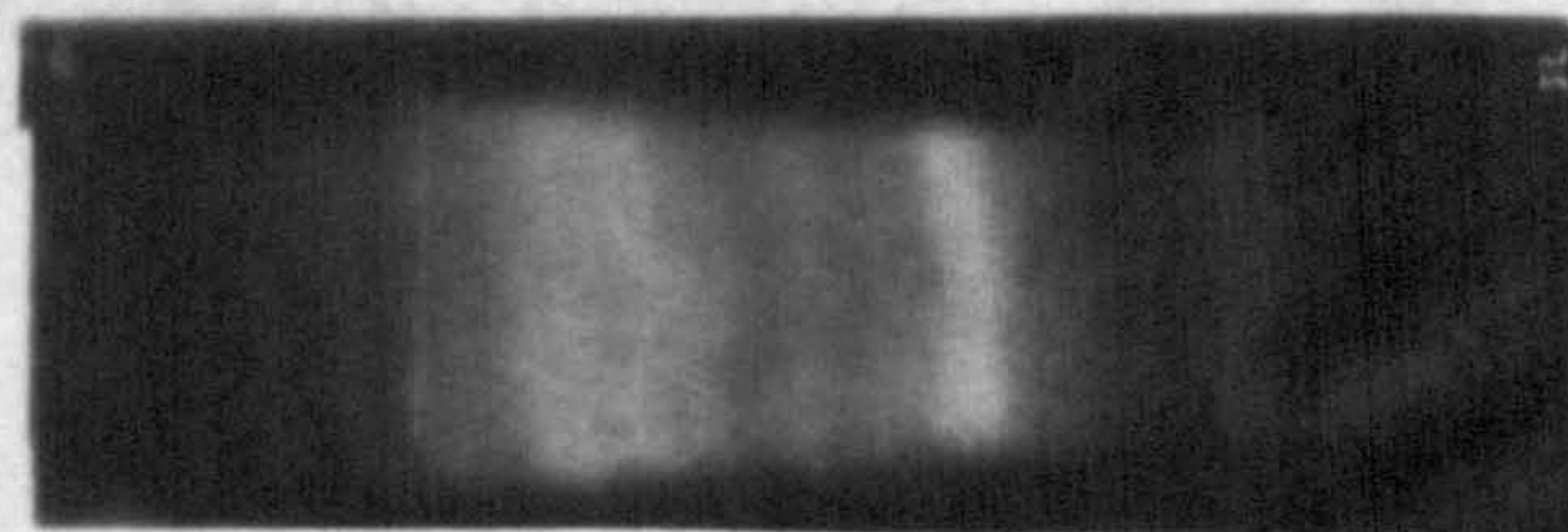
The appearance of blood fractions other than the respiratory pigment warranted further study, and blood from numerous specimens of the species examined in Experiment II was run, to discover the frequency of occurrence of these fractions, their variability within a single species, the ages of the snails in which they appeared, and to compare the proteins occurring in different snail species. During examination of the effect of pH on blood proteins of A. glabratus and P. corneus, the best separations of these protein bands were obtained with high pH buffers, and buffers pH 11.6-12.0 were used in general for this experiment. All snails were maintained in balanced, aerated aquaria at 80°F.

Results

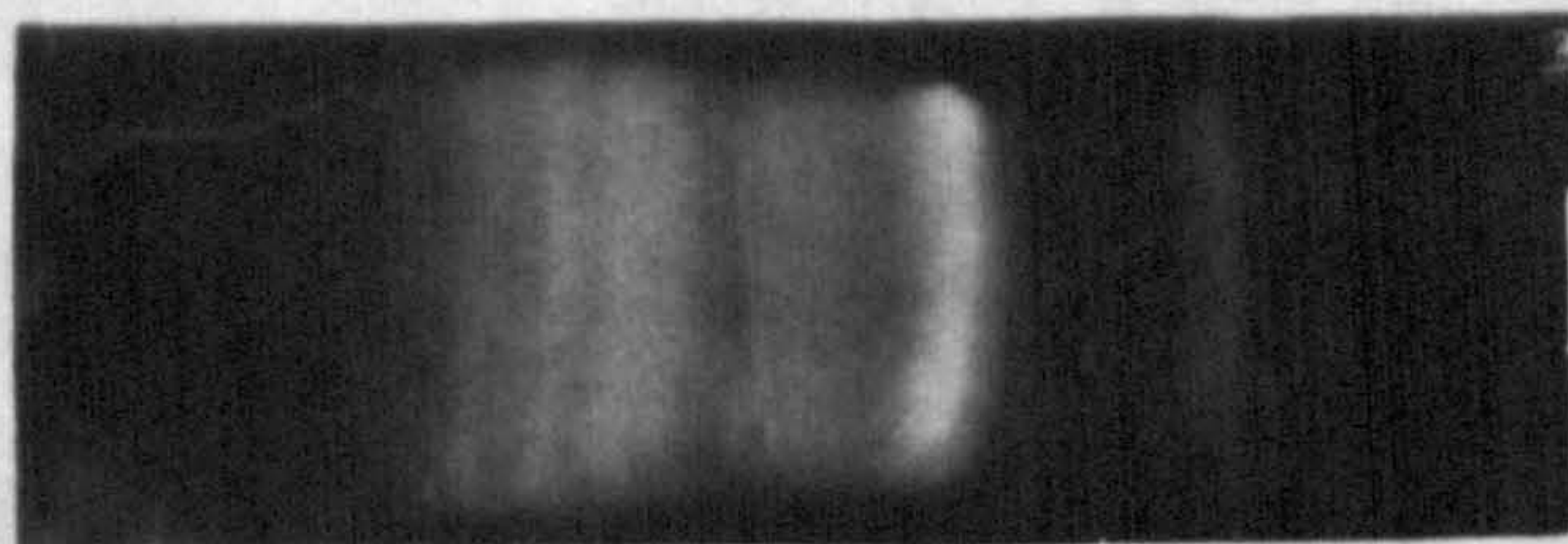
Figure 5 shows two patterns typical of those generally obtained with A. glabratus. Most snails again showed only the blood pigment protein but when other proteins occurred there were usually several fractions which were broadly similar in the different specimens. Figure 6 shows the relative amounts of the proteins obtained from four specimens of A. glabratus, the principal component being haemoglobin. Occasionally, samples showed rather indeterminate stained areas, with perhaps a single band

ELECTROPHORESIS OF SNAIL BLOOD PROTEINS

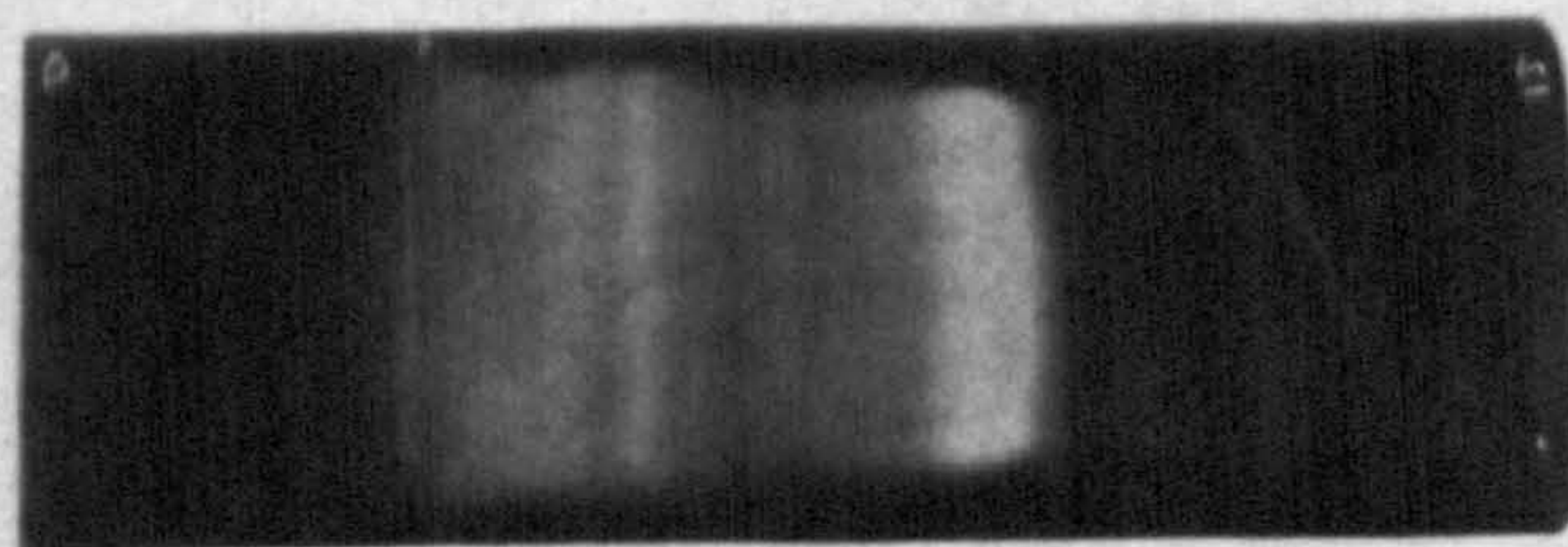
The Separation of Blood Proteins in Different Species



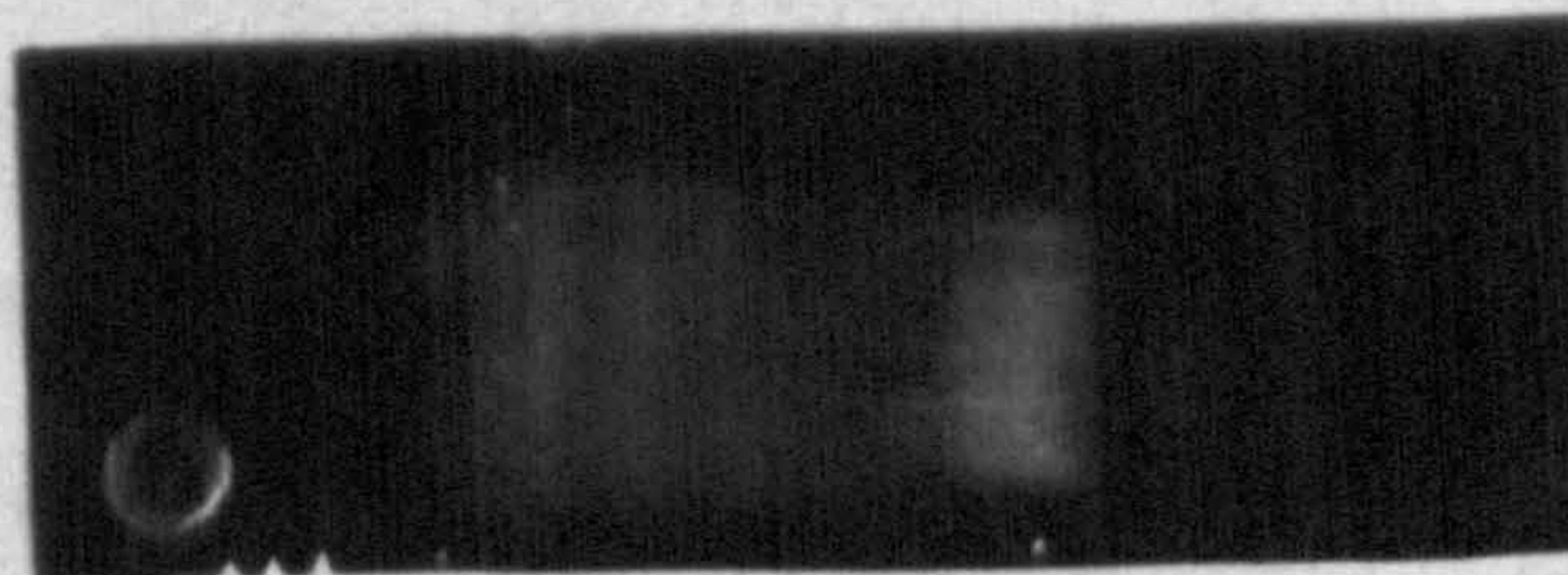
A. glabratus



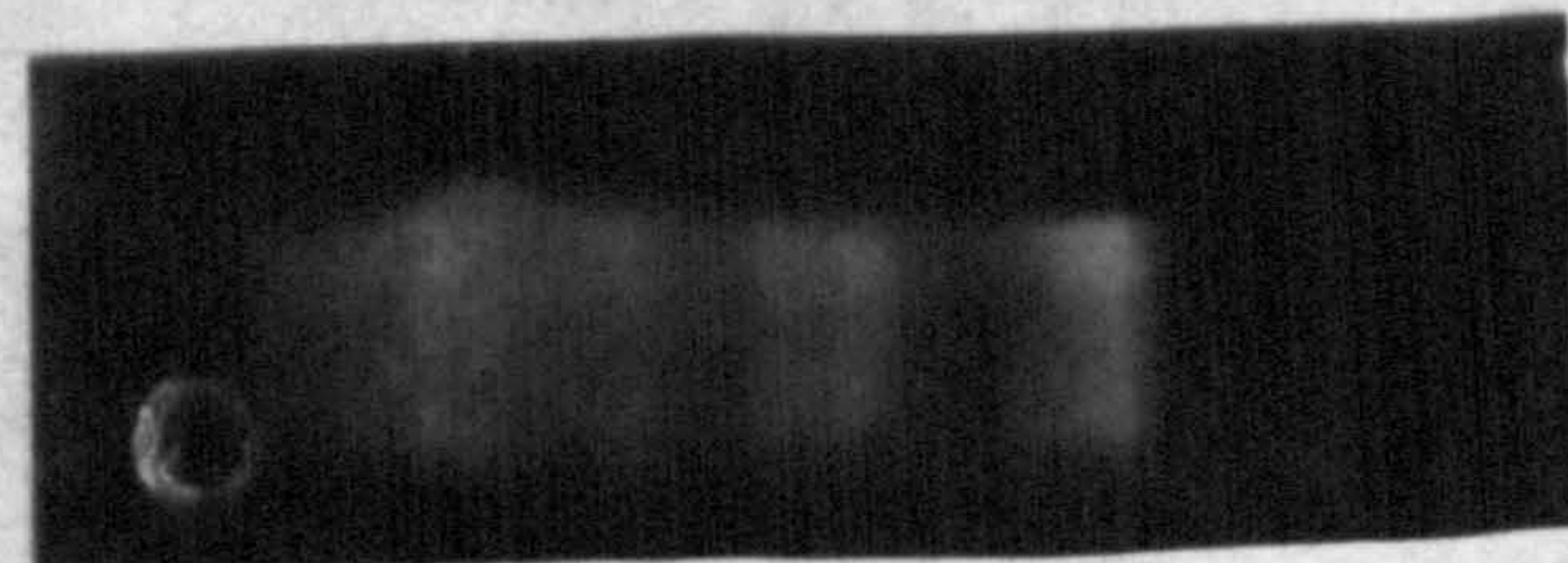
A. glabratus



B. truncatus



B(P) nasutus



L. stagnalis

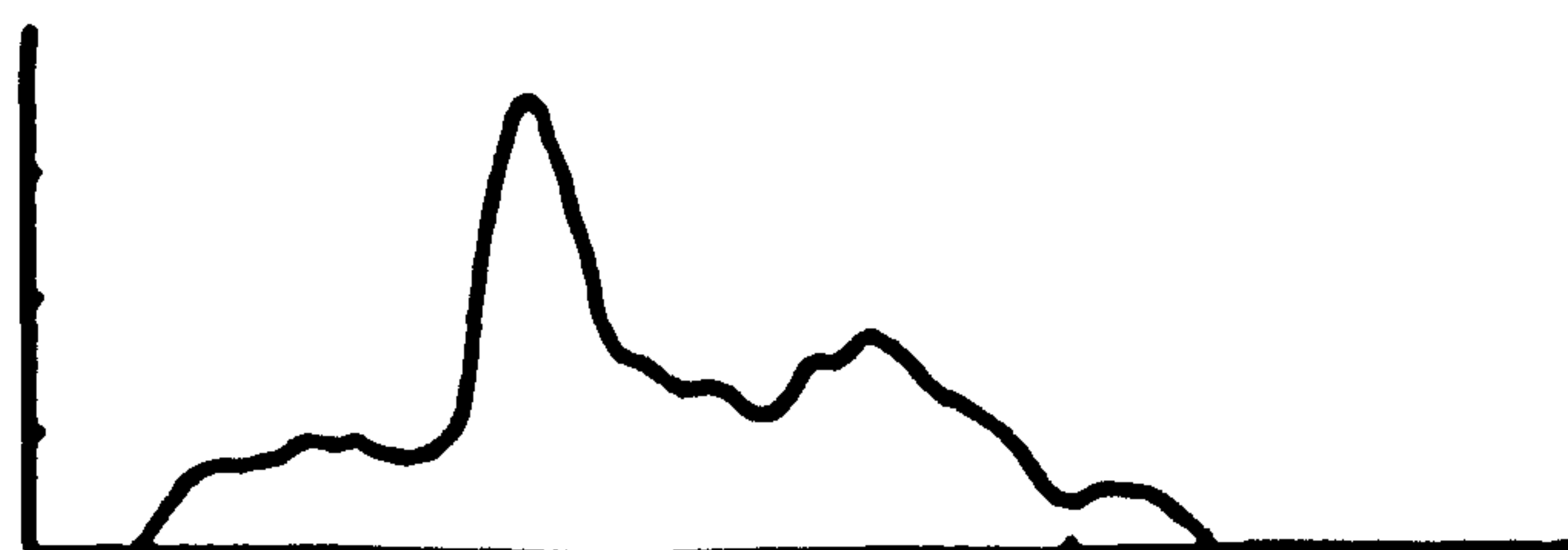
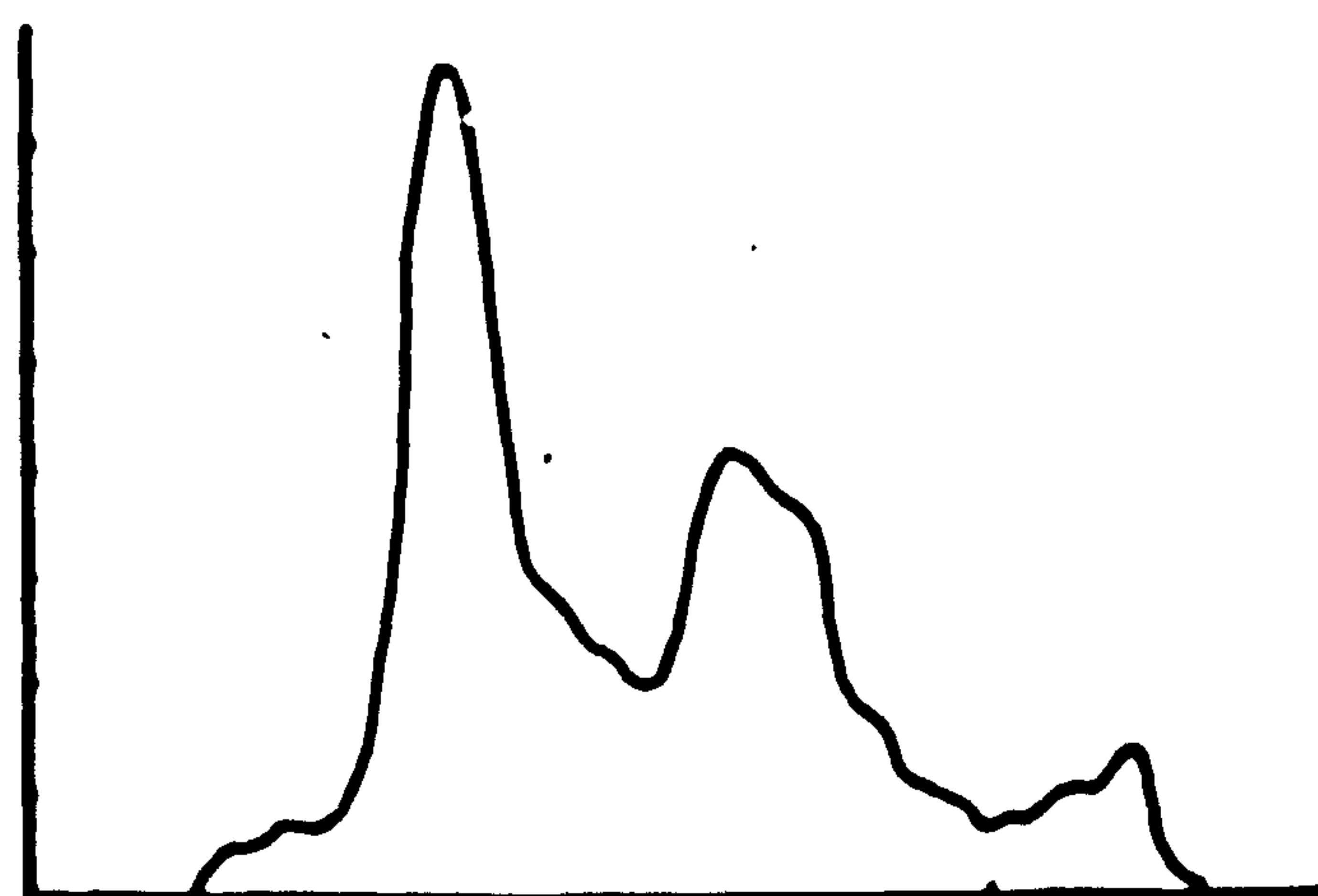
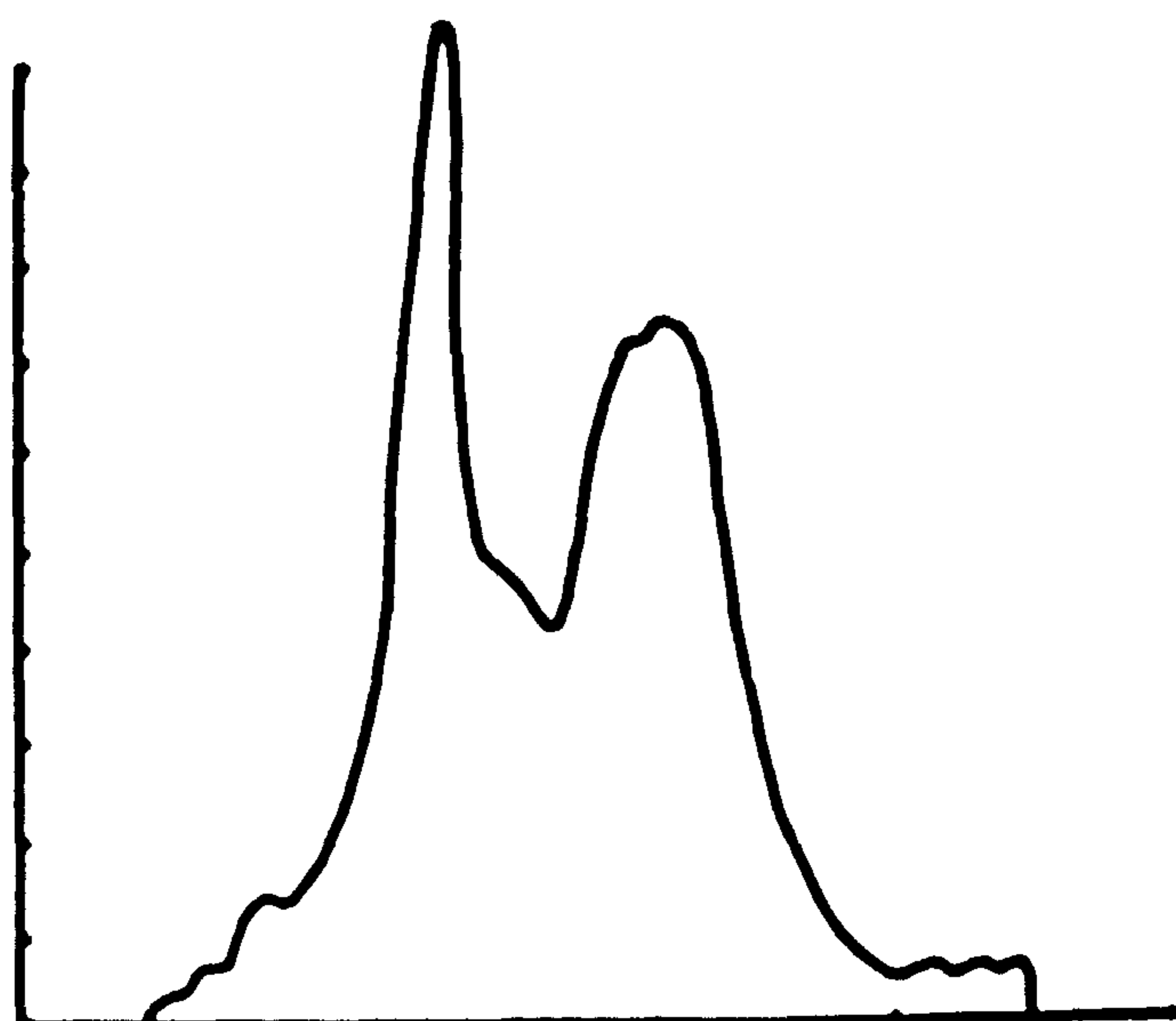


O. hupensis

FIGURE 5

SEPARATION OF SNAIL BLOOD PROTEINS

FIGURE 6

A. glabratusDENSITY

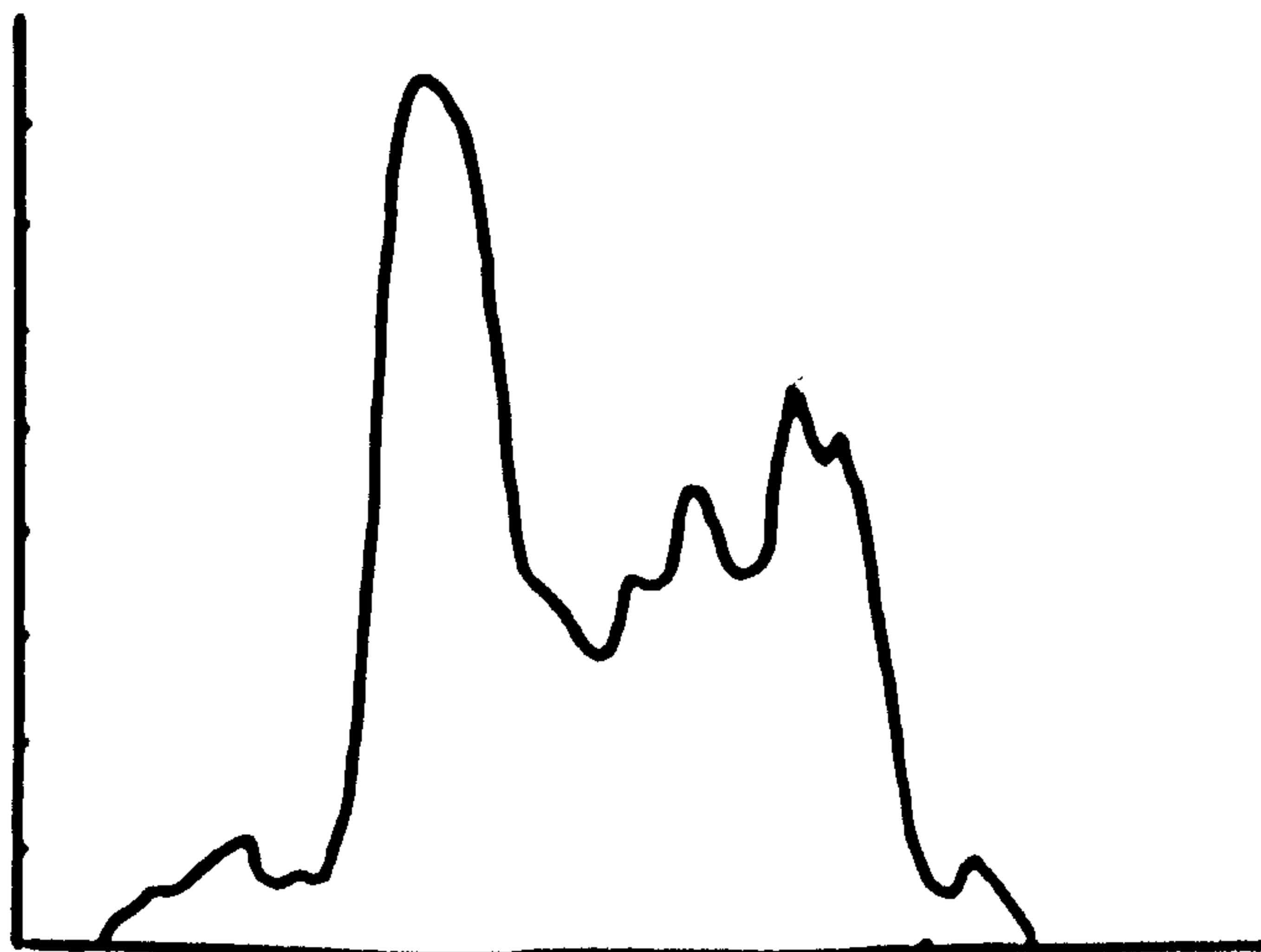
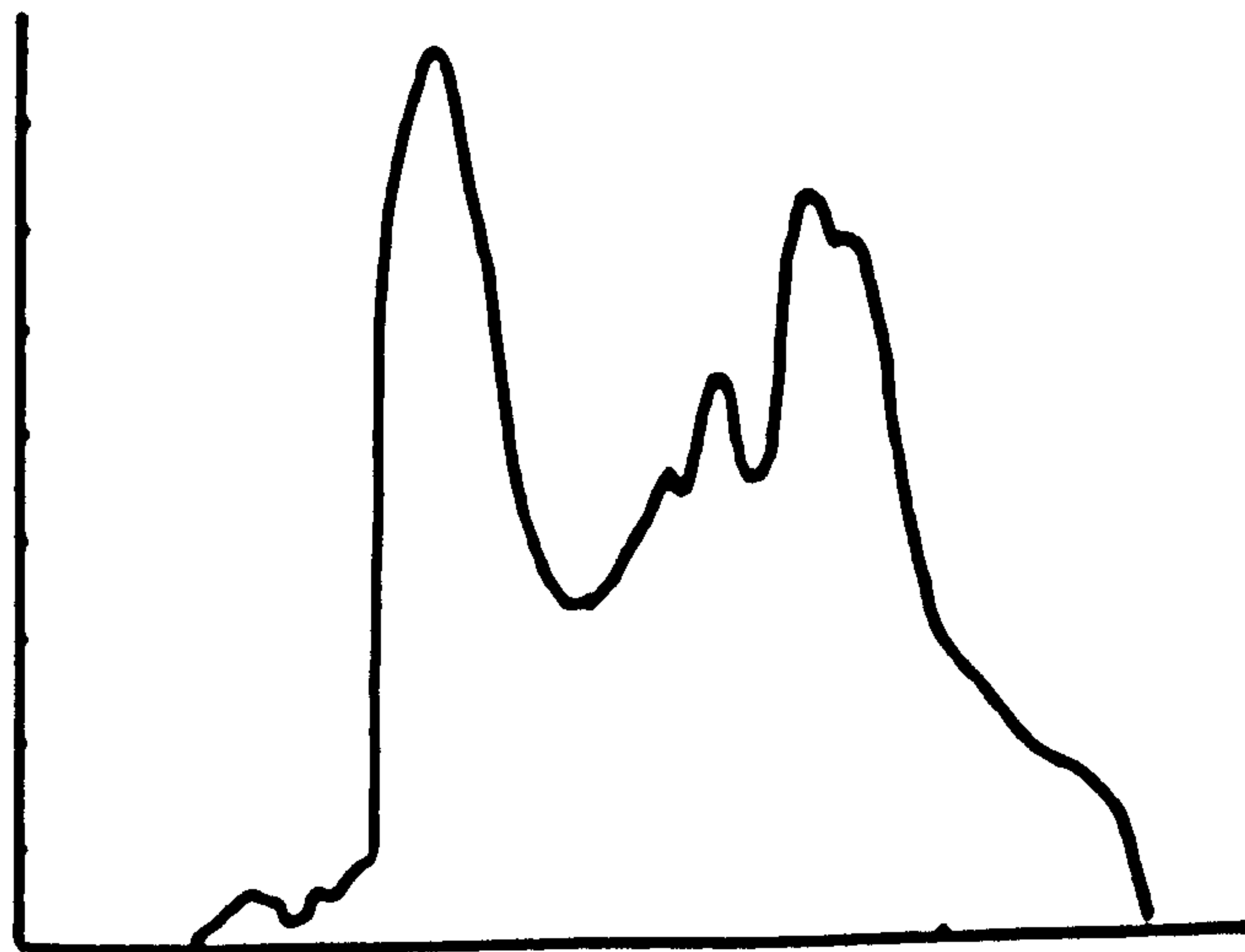
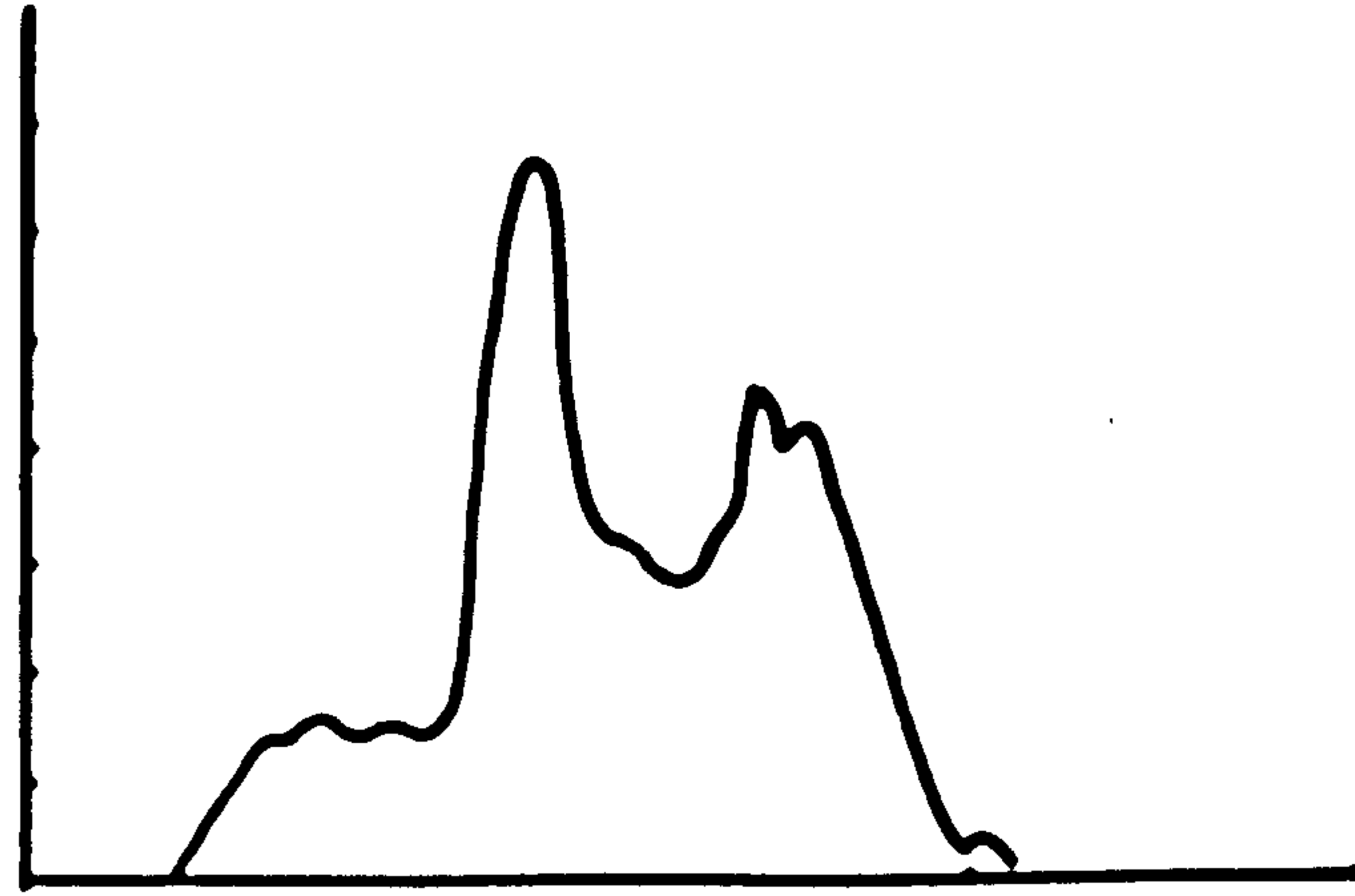
besides the haemoglobin visible, which bore no relationship to the normal pattern.

Haemoglobin was always the principal component and two other fractions which migrated more slowly than haemoglobin also showed regularly and in relatively high concentrations (Figure 6). The remaining bands were rather more variable in their occurrence and their concentration was generally low. Of the remainder, two or three fractions ran faster than the pigment, and one, two or three bands showed between the haemoglobin and the two common peaks. Up to five fractions with very low mobilities were obtained (Figures 5 and 6).

Snails varying between 3mm. and 17mm. in diameter were examined and these fractions only appeared in the blood of snails 3-12mm. in diameter, but most of the separations were obtained from the blood of specimens measuring 7-9mm. Blood samples from large numbers of B.(B). truncatus and P.corneus were run by the same method. Representative results of separations obtained with B.(B). truncatus are shown in Figures 5 and 7. Haemoglobin was again the only blood protein present in most samples but when separations occurred they were similar to those obtained with A. glabratus (Figure 7) although there was rather more variation in the relative concentrations of the different fractions. Haemoglobin

SEPARATION OF SNAIL BLOOD PROTEINS

FIGURE 7

B. truncatusDENSITY

was the principal component and the two fractions which were present in A. glabratus in high concentrations showed similarly with B.(B). truncatus, most variation occurring with the other proteins. A few specimens showing diffuse areas without definite bands were obtained.

Three specimens of P. corneus showed separations which seemed related to the patterns obtained with the other snails (Figure 8).

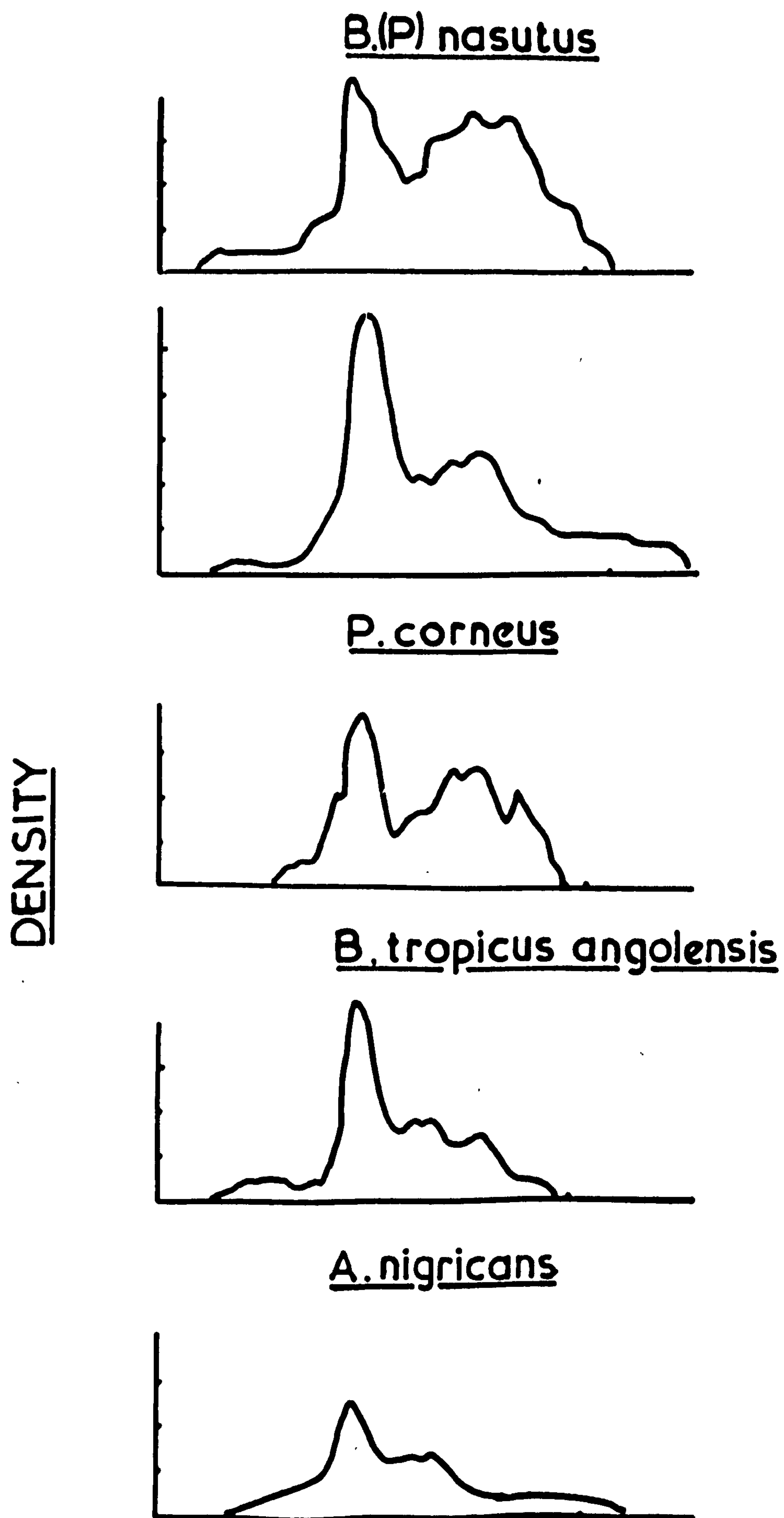
Small numbers, ten to twenty specimens, of the other planorbidae were examined and separations obtained with two specimens of B.(B). tropicus angolensis and of B.(P). globosus and single specimens of A. nigricans and T. centimetralis. Figure 8 shows patterns obtained with B.(B). tropicus angolensis and A. nigricans. None of these matched the basic separation common to A. glabratus, P. corneus and B.(B). truncatus and they showed poor separation of bands against a stained background.

Fifteen specimens of B.(P). nasutus were examined and ten of these contained blood proteins. These varied in concentration and mobility but most of them could be related to the pattern commonly obtained (Figures 5 and 8).

The range of sizes of the different species examined and the size range in which separations were obtained were as follows:-

SEPARATION OF SNAIL BLOOD PROTEINS

FIGURE 8



<u>Species</u>	<u>Range of sizes⁺ examined</u>	<u>Range of sizes at which separations were obtained</u>
	<u>mm.</u>	<u>mm.</u>
1. <u>A. glabratus</u>	3.0 - 17.0	3.0 - 12.0
2. <u>A. nigricans</u>	7.5 - 13.5	9.0
3. <u>B. s. tanganyicensis</u>	10.5 - 13.5	-
4. <u>B. p. ruppellii</u>	5.0 - 10.5	-
5. <u>T. centimetralis</u>	4.5 - 5.5	4.5
6. <u>P. corneus</u>	5.0 - 17.0	8.0 - 14.5
7. <u>B. (B). truncatus</u>	4.5x2.5 - 9.0x5.0	5.0x2.5 - 7.5x4.5
8. <u>B. (B). t. angolensis</u>	4.0x2.5 - 6.0x4.0	5.5x4.0
9. <u>B. (P). nasutus</u>	7.5x4.0 - 13.0x7.5	7.5x4.0 - 13.0x7.5
10. <u>B. (P). globosus</u>	4.75x3.0 - 12.0x7.0	8.0x5.0 - 12.0x7.0

Comment

Blood proteins other than haemoglobin appeared in only small numbers of each species except B. (P). nasutus, where a high proportion showed additional bands. While concentrations of the additional fractions varied within and between species, the typical patterns obtained with

⁺ Flat snails were measured at the greatest diameter. Figures for snails with a spire are the height and greatest diameter.

A. glabratus, P. corneus, B.(B). truncatus and B.(P). nasutus were similar, which suggests that the comparable bands represent the same or similar proteins. One or two specimens of each species, however, did show definite stained areas, but little or no separation into bands, thus differing from the usual pattern. Protein bands occurring in A. nigricans, B.(B). tropicus angolensis (Figure 8), T. centimetralis and B.(P). globosus did not match this pattern, but resolution was poor and further examinations are necessary before direct comparisons can be made with other species.

Data of the sizes of snails which showed separations are included because of the restricted size range, 7-10 mm. diameter, within which protein separations were obtained by Wright and Ross (1959) with A. glabratus.

No definite relationship was indicated between the size of the snails and the appearance of blood proteins for the majority of the species examined, since there was not enough material available to make a representative examination of each species. Where sufficient numbers of snails were examined, as in the case of A. glabratus, P. corneus and perhaps B.(B). truncatus, most separations occurred with medium sized snails, 7-9mm. diameter for A. glabratus and 8-12 mm. for P. corneus. This is similar

to the results obtained by Wright and Ross (1959) with A. glabratus although the overall size range in which additional proteins were obtained was greater than that reported by these authors. Large specimens of A. glabratus and P. corneus never showed these proteins or any suggestion of additional fractions, but again it was not possible to decide from the snails examined whether this was true of all the species.

Since the patterns obtained with four species showed similarities, it seems probable that they appeared in response to a common stimulus. Although there was considerable variation within this basic pattern in each species, they were sufficiently alike to suggest a common origin. The interpretation of results which did not match this pattern is more difficult, especially since there were only a few samples showing the separations. Since four species, which are quite distinct by taxonomic classification, showed a similar pattern generally, the less definite separations obtained from these snails, and possibly from the other species, may represent the first appearance or last remains of the non-haemoglobin fractions.

No determinations were made of the length of time these proteins remain in the blood but it is probably several days at least. One specimen of A. glabratus in which the

proteins occurred showed similar patterns on three successive days. Only one example of the disappearance of the bands was obtained. A specimen of A. glabratus examined about six weeks after the bands had been seen showed only the haemoglobin protein.

The factors which govern the appearance of these proteins were not obvious from these experiments. All snails were kept under normal laboratory conditions in balanced aerated aquaria so that the stimuli for the formation of the bands remained constant.

The bands only appeared irregularly in snails which were presumably growing actively and laying eggs. The common pattern shown by four species and the low rate of occurrence of the patterns suggest that their appearance is related to an irregularly occurring vital function - for example egg laying - which is common to all the snails.

Experiment IV

Electrophoresis of Blood from Snails Maintained Under Different Environmental Conditions

All snails were normally maintained in glass aquaria, 14" x 10" x 9", containing filtered pond water and a thin bottom deposit of coarse sand. The tanks were planted with broad leaved Ludwigia palustris, and some contained, in addition, Vallisneria spiralis and Myriophyllum sp. The aquaria were aerated and snails fed with an alginate food (Standen, 1951).

Four batches of A. glabratus were maintained under different conditions in an attempt to induce the appearance of blood proteins other than haemoglobin.

Batch A

A mouse jar was filled to half-an-inch with coarse sand, half-an-inch of fine soil added and the jar three parts filled with filtered pond water. Twenty-five snails, 4-12mm. in diameter, were added and the jar kept in a dark cupboard. This was considered an unbalanced environment with no plants, aeration, light or food. The snails were examined daily, any snails out of the water being pushed back. Most of them remained near the surface but although the water became turbid after five days, no deaths occurred. After seven days, sixteen snails selected at random were transferred to a balanced tank which contained plants and was aerated and

illuminated. Food was added on the second day after transfer.

Egg masses appeared in the balanced tank twelve hours after adding the snails.

Batch B

Forty snails, 4-15 mm. in diameter, were kept out of water in moist conditions. Half were placed in a large petri dish containing damp filter paper, and the lid arranged to leave a small air space. The remaining snails were placed between layers of damp cotton wool in a tin containing air holes.

They were examined daily and after seven days ten snails from each group were transferred to new balanced tanks. Snails kept in the petri dish became fully retracted and it was difficult to decide whether they were alive. Three of the ten snails transferred from the dish to a balanced tank were dead. The first egg mass appeared two days after the transfer and several more appeared later. Snails kept in cotton wool were retracted slightly but were obviously alive. The snails from this group which were added to a breeding tank also produced egg masses after two days.

The remaining snails kept on filter paper and

cotton wool were bled after eight days and then pooled in a balanced aquarium.

Batch C

A normal breeding tank, 14" x 10" x 9", was allowed to become overcrowded. It contained 200-300 snails but conditions were otherwise normal.

Batch D

Two control breeding tanks were prepared, each containing 40 snails 5-10 mm, in diameter, with adequate supplies of plants, light and food. The tanks were aerated. Egg masses appeared within twenty-four hours.

Electrophoretic examinations

Snails were examined by electrophoresis of the blood at the following stages:-

1. Snails maintained in the mouse jar for eight days, in poor conditions, were examined and then discarded.
2. Snails kept in the mouse jar for seven days and then transferred to a balanced tank were examined three days after the change to balanced conditions when they were breeding well.
3. The batches of snails kept in cotton wool and on filter paper and then returned to balanced tanks were examined when breeding commenced (Two days).
4. Snails kept for eight days on cotton wool and

filter paper were examined and then pooled in one balanced tank. All the snails survived and further blood samples were taken 3, 5, 7, 10 and 14 days later. Seventeen of the twenty snails survived to the last examination and egg masses appeared on the second day and between the seventh and tenth days.

5. The control breeding snails and those from the over-crowded tank were examined frequently alongside the other experiments.

Results

No separations were obtained with any of the samples examined. The amount of haemoglobin was higher in snails kept out of water or in the unbalanced conditions of the mouse jar but there was no sign of other proteins.

Comment

The number of snails in each batch was small but it was considered that if the stimulus for the appearance of the blood proteins occurred it would show with even relatively few snails. In the previous experiment a single snail showed blood proteins on three successive days, and it is, therefore, unlikely that they could have been missed in these examinations.

Snails maintained under abnormal conditions have to suspend or greatly modify many normal physiological

functions, and the purpose of these examinations was to determine whether conditions of stress would influence the appearance of the blood fractions or whether they would appear when the normal physiological state was re-established.

Egg-laying commenced within two days of snails being returned to balanced aquaria so that presumably all other normal functions were quickly re-developed.

The blood proteins appeared in snails from established breeding tanks, yet none of the eighty snails transferred from these tanks to new balanced aquaria showed the separations although they bred well. The appearance of the additional lines, therefore, was not induced during the period of starting to lay eggs or when the snails were subjected to drying, starvation, and absence of light or aeration. It may be that these blood proteins are related to a more long term effect which might not show in this short experiment.

Experiment V

Electrophoresis of Blood from *Lymnaea stagnalis* and *Oncomelania hupensis*

Blood was examined from two snails which do not contain haemoglobin, for comparison with the planorbidae.

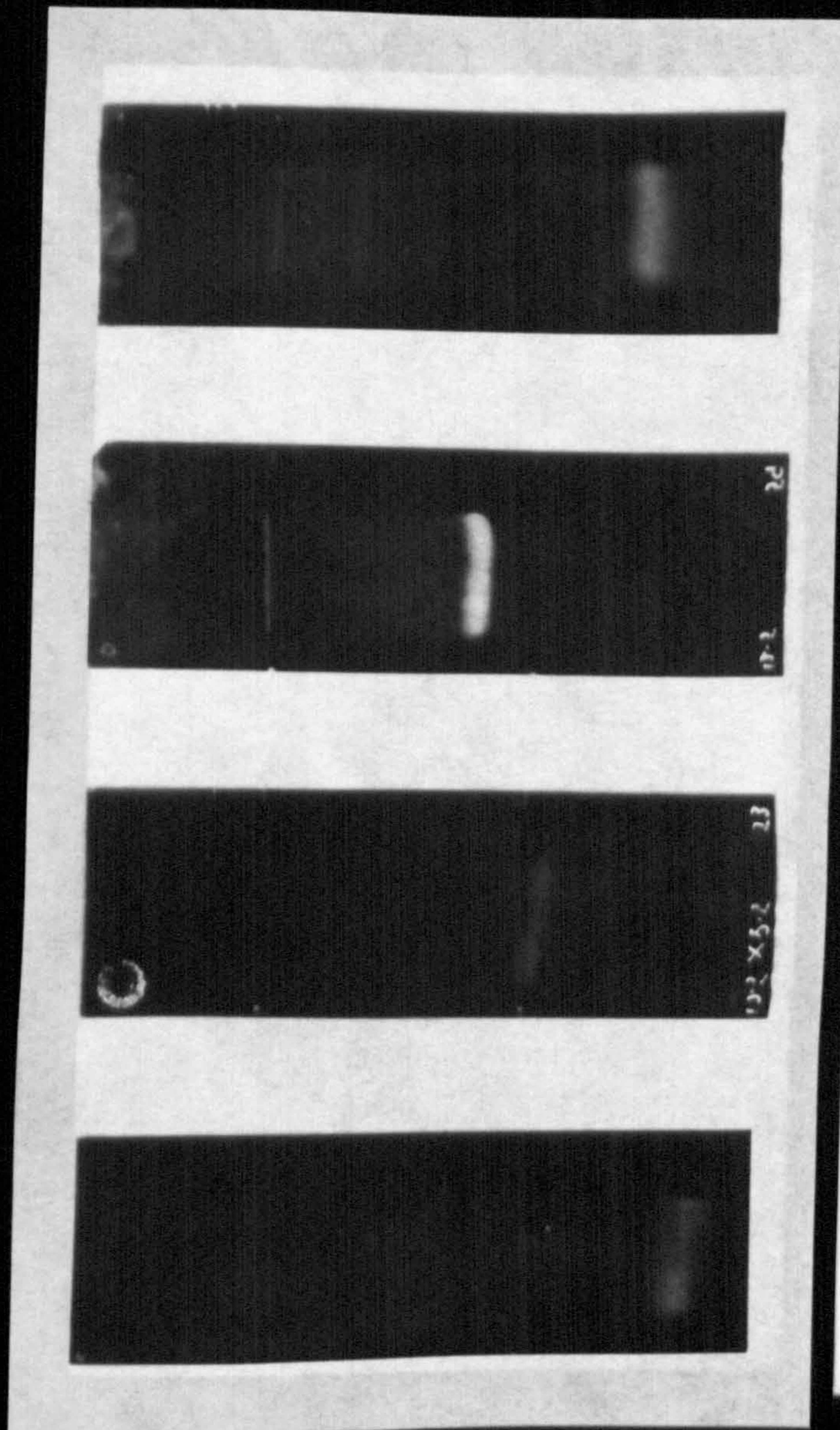
Numerous specimens of *Lymnaea stagnalis*, which contains a haemocyanin blood pigment, were examined at pH 8.6 and 11.6 (Appendix II, Nos. C1 and C2). The snails ranged in size from 9.0 x 3.5 mm. to 35.0 x 19.0 mm. Blood from a small number of *Oncomelania hupensis*, which is believed to contain a haemocyanin pigment, was examined in a similar way. Blood was obtained from both species by piercing the tip of the shell spire.

Results

At pH 11.6, blood from *L. stagnalis* showed a single band, the haemocyanin, which moved slightly faster than snail haemoglobin, generally without tailing (Figure 9). At pH 8.6 the rate of movement of the haemocyanin was similar to that with the more alkaline buffer but the band was rather more diffuse although there was usually no tailing to the application point. A few samples showed lightly stained areas ahead and behind the haemocyanin but no definite bands occurred. In one specimen, however, separation of blood proteins did occur (Figures 5 and 10)

ELECTROPHORESIS OF BLOOD PROTEINS ⁶⁴

Comparative Rates of Movement of Serum Proteins and Snail Blood Pigments



Baboon Serum

A. glabratus
Haemoglobin

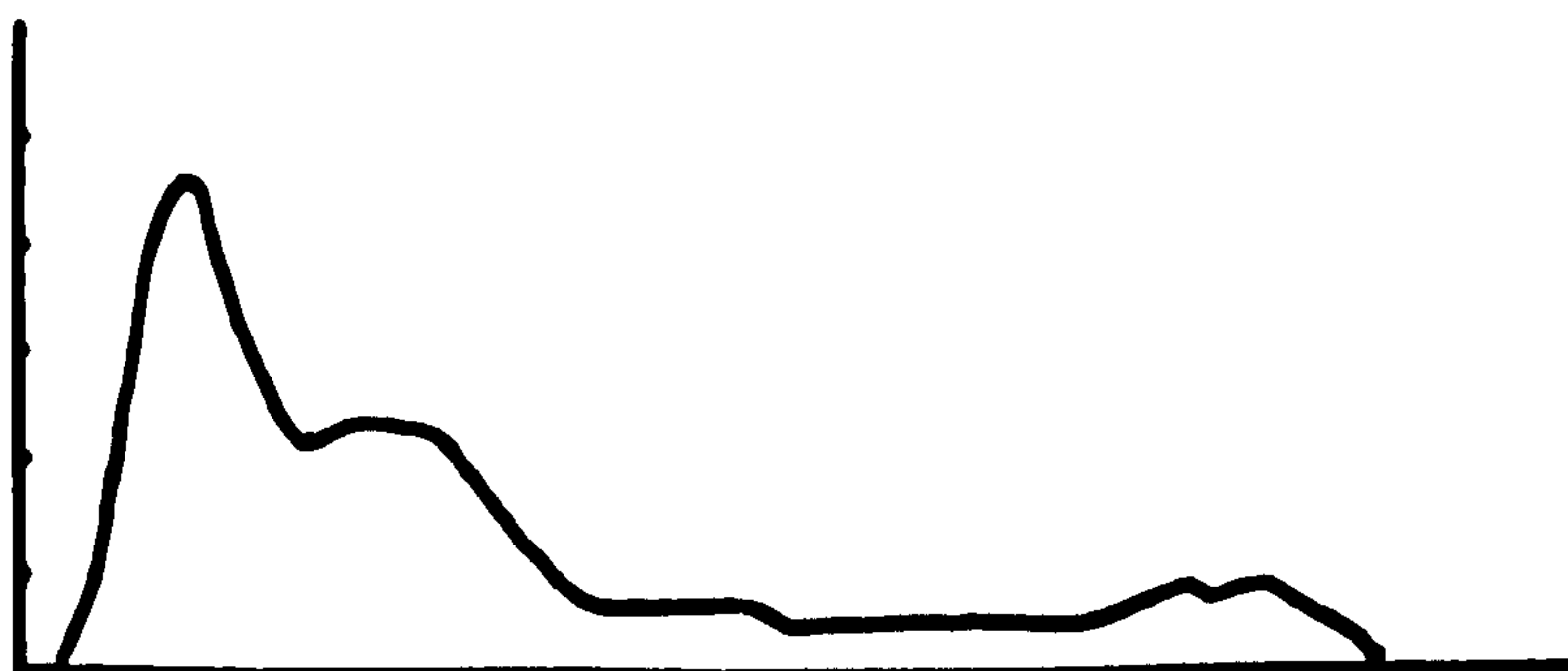
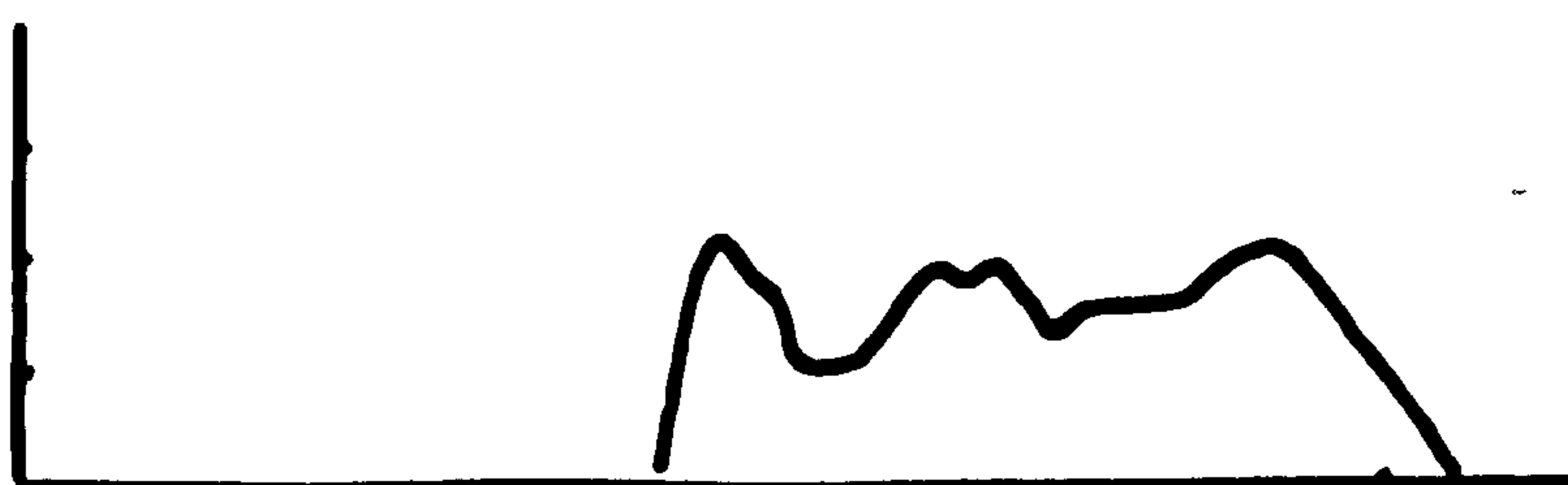
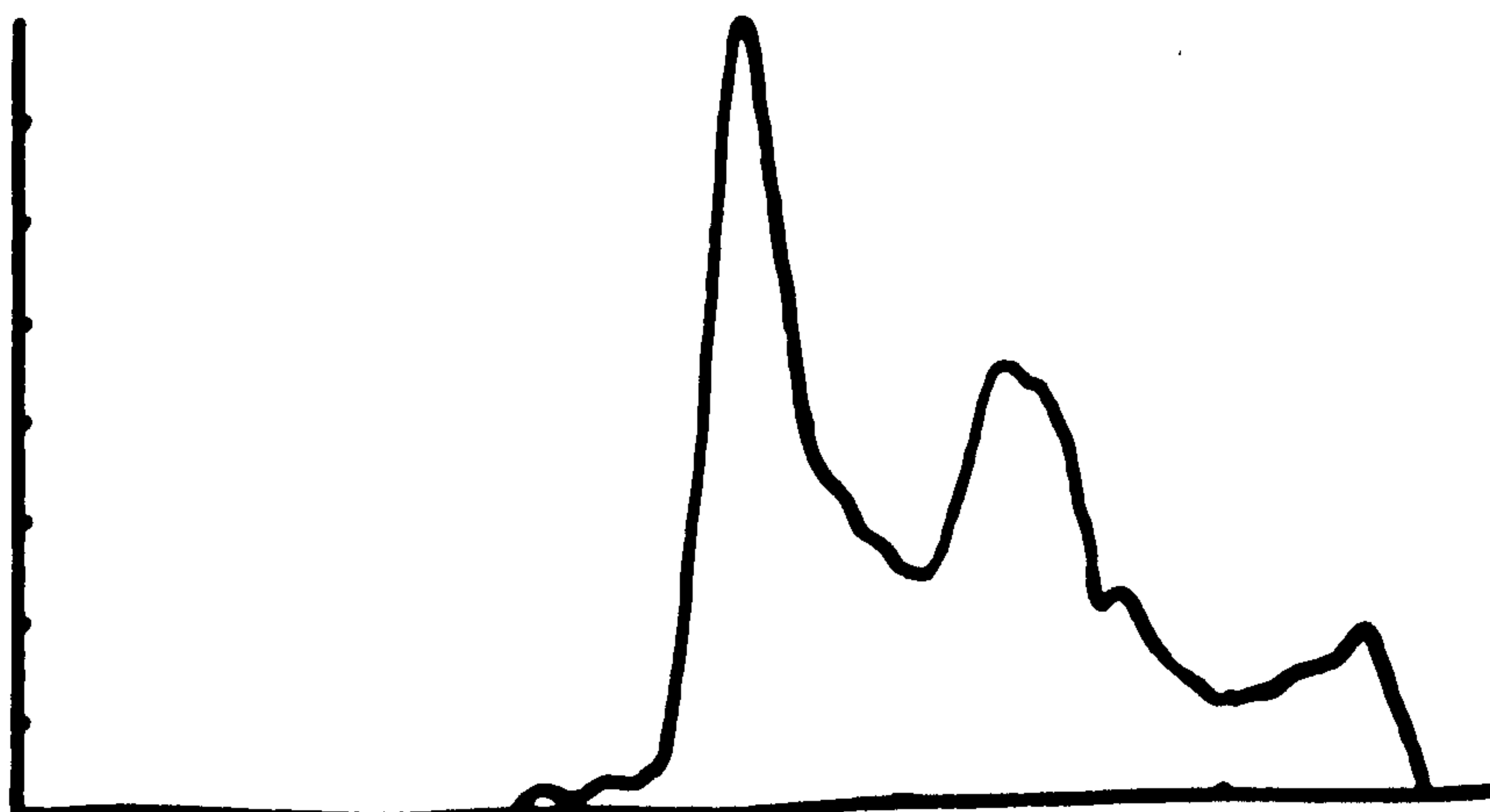
L. stagnalis
Haemocyanin

Q. hupensis
Haemocyanin?

FIGURE 9

SEPARATION OF SNAIL BLOOD PROTEINS

FIGURE 10

O. hupensisL. stagnalisA. glabratus

the patterns being similar at pH 8.6 and 11.6.

O. hupensis also showed only one blood protein, the pigment protein, in all but one of the specimens examined. This pigment behaved similarly with both buffer solutions, and the rate of movement was considerably greater than that of haemocyanin from L. stagnalis and slightly greater than that of serum albumen (Figure 9). One snail showed rather poor separation of fractions other than the pigment protein (Figures 5 and 10).

A representative separation from A. glabratus is included in Figure 10 for comparison.

General Comment

In experiments I to IV it has been shown that the haemoglobins of different snail species are similar to one another with regard to their electrophoretic mobility. The pigment protein behaves in the same way at different pH levels, forming a distinct band, which suggests that, with regard to proteins alone, the blood is a pure solution of haemoglobin. The tailing that occurred was almost always small and seemed to be due to the haemoglobin molecule. Thus, when a single sample of blood was examined in different experiments the amount of tailing varied more than would be expected if another protein were present. In another experiment, different quantities of blood from A. glabratus were applied to the strips and the movement of the haemoglobin followed

during irrigation. The haemoglobin is visible without staining and with higher concentrations of blood the colour was visible behind the main pigment band. The amount of colour decreased towards the point of application; the same pattern was obtained by scanning stained preparations. This again supports the view that the tailing was not due to a second protein; it may well be connected with the large size of the molecule.

Evidence has been cited (page 15) that blood proteins act as buffers. It has also been shown that haemocyanin provides blood colloid, and helps to maintain equilibrium with the environment since the haemolymph is under hydrostatic pressure (Redfield 1934). These experiments have shown that the snails with a haemocyanin pigment generally contain only the pigment protein in the blood and it seems probable that the snail haemoglobins and haemocyanins would function in a similar way as blood colloids.

The buffering capacity of proteins depends on the acid-binding groups of the molecule (Prosser et al 1952). In conjugated proteins which contain an iron-porphyrin group, such as haemoglobin, differences in the chemical nature and physiological role are largely due to differences in the protein parts of the molecules and in the nature of the linkages which unite the protein portion, or globin, with the prosthetic group (Fruton and Simmonds 1956).

It appears therefore that the haemoglobin influences the internal environment of the snail and perhaps characterises the physiological norm in this group of snails.

The appearance of protein fractions other than the pigment in the blood seems to suggest that a snail produces additional proteins, at infrequent intervals, in response to some particular physiological state of the animal. Demonstration of these fractions in L. stagnalis and O. hupensis, as well as in the snails containing haemoglobin, shows that they correspond to a function common to all the snails. This is further indicated by a general similarity in the patterns commonly obtained with four snail species. Since these bands appeared in snails of different ages it is unlikely that they represent a particular phase in, for example, the growth of the animal, but the experiments gave no indication of other stimuli which might have induced their formation.

These additional fractions may have been responsible for the appearance, in the spectrophotometric analyses, of the small extra peaks which appeared occasionally, since blood for these examinations was obtained from snails of differing sizes and included those which could have shown the proteins.

It is doubtful whether electrophoresis will yield much further information about the pigment proteins but it

might determine the nature of the other blood proteins and the factors governing their appearance.

C. The Analysis of Snail Blood Amino Acids
 by Paper Chromatography

Introduction

Protein solutions show both chemical and colloidal properties, the former being due to the presence of free amino and carboxyl groups in the molecule which allows them to function as amphoteric electrolytes, and the latter being governed by the large size of the molecule and the degree of charge or hydration of the particles (Hawk et al 1954). The nature and inter-relationships of these reactions are largely unknown in invertebrates but the preceding studies have shown that these properties of the snail blood pigment proteins are particularly important to the maintenance of the internal environment since the pigment is commonly the only protein present in the circulatory system. The amino acid constituents of the protein portions of these molecules were, therefore, examined to determine whether different haemoglobin types occurred in the planorbid snails, differences might be found which indicated variation in the properties of the molecules, or whether there was a common pattern in the five species examined.

The free amino acids of the blood were also examined to determine whether any qualitative differences

could be found which might affect the ability of snail species to support schistosomes.

1. Amino Acids of Snail Blood Pigments

a) Preparation of samples

Blood was obtained from the snails by the technique already described. Large specimens of each species were used, as far as possible, since these contained no blood proteins other than the pigment protein. The blood samples were examined electrophoretically, however, to ensure that no other fractions were present. The pattern obtained, and already described, from snails containing haemoglobin was a strong haemoglobin band with slight tailing to the origin. It has been shown already that the tailing was almost certainly due to the haemoglobin. Since the amount of material forming the tail was small, generally about five per cent of the total, the haemoglobin was considered pure if no suggestion of other bands was obtained.

Haemoglobin was precipitated from pooled blood samples by addition of 40% ammonium sulphate solution. Approximately two volumes of ammonium sulphate were required to ensure complete precipitation. A small amount of mucoid material often floated on the surface after precipitation and this was removed with the supernatant, which contained any free amino acids. The haemoglobin was dissolved in a small quantity of water,

generally 1-2 ml., and dialysed in a refrigerator against distilled water for 10-14 days, the water being changed daily. This removed all inorganic salts. The protein usually remained wholly in solution but occasionally a small amount precipitated in the dialysis sac. This was discarded and the solution evaporated to dryness. During early experiments, electrophoresis of the blood was performed after dialysis but the pattern was the same as that obtained with fresh blood and in later experiments electrophoresis was discontinued. The haemoglobin was weighed in an ampoule. 6N hydrochloric acid was added to make an approximately 1% solution, the ampoule sealed, and the protein hydrolysed at 120°C for 24 hours. In all examinations prior to those used with the final technique, the hydrolysed sample was centrifuged, the supernatant removed, the residue washed and the filtrate added to the first supernatant and evaporated to dryness. The residue, after evaporation, was washed with distilled water and evaporated again, this process being repeated three or four times. The dry sample was finally dissolved in 10% aqueous iso-propanol to give a solution containing approximately 10 mg./ml., related to the weight of haemoglobin before hydrolysis. For the final technique, the hydrolysed sample was centrifuged and the supernatant retained. This was yellow or brown

in colour and this acid solution was passed through a Dowex 50 resin column. Much of the colour passed through the column directly. The resin was washed well with water and the amino acids eluted with 10% ammonia solution. The remaining colour was eluted at the same time but when the ammoniacal solution was evaporated to dryness and 10% iso-propanol added, the coloured material was normally insoluble. If the final solution still contained any colour, it was passed through a resin column a second time, evaporated to dryness and the amino acids re-dissolved in 10% iso-propanol.

The haemocyanin pigment of L. stagnalis was treated in the same way to obtain the amino acids of the protein portion of the molecule.

Alkaline hydrolysates were prepared by hydrolysing the blood pigments with 0.38 N barium hydroxide for 24 hours at 120°C. The amino acid solution was neutralised by adding 2N sulphuric acid drop by drop, spinning the solution between additions, until no further precipitate of barium sulphate was obtained. The solution was then treated in a similar way to the acid hydrolysate.

Materials

Qualitative examinations were made of the amino acid composition of haemoglobin from A. glabratus, P. corneus,

B.(P). globosus and B.(P). nasutus and of haemocyanin from L. stagnalis. Quantitative estimations were made with extracts from A. glabratus, P. corneus and B.(P). nasutus.

b) Preliminary Experiments

Three chromatographic techniques for separation of amino acids were tried to determine which would be most satisfactory for these examinations.

1). Two-dimensional ascending chromatography

The apparatus consisted of sheets of chromatography paper, 15 cm. sq., fitted into a frame, the lower edges of the papers dipping into a tray containing solvent, the whole being contained in a glass tank with a lid. Two solvent systems were used:

1. 250 ml. of phenol were shaken with 62.5 ml. of water. A small quantity of sodium cyanide, in 5 ml. of water, was placed in the tank in a beaker; this prevented decomposition of the phenol. 0.3% ammonia solution, which aided the movement of basic amino acids, was added in a second beaker.
2. 200 ml. of 1-butanol was shaken with 200 ml. of water and 48 ml. of acetic acid. Two layers

formed, the upper one being used as the moving phase with an aliquot of the lower layer in a beaker in the tank.

The papers were stained with 0.25% w/v ninhydrin in acetone.

Samples were applied 2.5 cm. from the lower and left hand sides. Up to twelve papers were run in a single experiment. The phenol-water solvent was used in the first direction, the solvent front being allowed to run to the top edge of the paper. Papers were dried at room temperature and then irrigated in the second direction using the butanol-acetic acid-water solvent, the front again being allowed to reach the upper edge of the paper. The papers were dried at 75°C for at least one hour before staining.

Results

Samples of haemoglobin amino acids from A. glabratus were examined alongside standard amino acids. The blood samples showed irregular diffuse spots some of which did not separate well from one another. Between these spots, lightly stained areas occurred and it was difficult to decide whether they represented amino acids or not as they were not distinct areas.

It was considered that even with improved resolution, separation of a large number of amino acids would not be practicable on this comparatively small paper. When the amino acid spots are so close together, any small variations between the rates of movement on the different papers containing standards and samples would make interpretation of the results difficult. The method was therefore abandoned.

2) Single dimension ascending chromatography

a) Single development irrigation was used with various sized papers. A glass tank 30 x 25 x 36 cm. was fitted with glass rods 30 cm. long. Solvent was placed in a large dish in the bottom of the tank. The position of the glass rods was adjusted according to the length of paper used, so that the papers dipped into the solvent. The papers were stapled to the rods. Sheets of paper 20 cm. wide and 15, 30 and 33 cm. long were used. Samples were spotted 2.5 cm. from the bottom of the paper and 2.5 cm. apart. Strips of paper 30 x 3 cm., each containing a single sample, were also used.

The solvent used was n-butanol-acetic acid - water 450: 50:125. The solvent front reached 13-15 cm. with the short paper and between 22 and 28 cms. with the longer

strips.

Results

The differences in Rf values of the amino acids used were small and separations with mixtures of known amino acids and with blood samples of A. glabratus were poor in consequence. With the longest run the spots were more diffuse and 25 cm. seemed to be the maximum length of run.

b) Sheets of chromatography paper 15 and 30 cm. long were developed to 14 and 25 cm. respectively. The papers were irrigated three times in the same solvent.

Results

The patterns were an improvement over the single development and with blood samples of A. glabratus up to 16 spots were visible although they were not well separated. The 30 cm. sheets were not a great improvement over the 15 cm. sheets as the spots were larger and rather diffuse. The technique was finally abandoned with the development of a satisfactory method of separation on a horizontal circular apparatus.

3). Kawerau Circular Chromatography Apparatus

This is commercially produced (Shandon Scientific Co.) and consists of a shallow circular dish with a ground

glass flange. Solvent is fed to the paper through a glass capillary tube which is centred in a groove in the dish. The paper is 26 cm. in diameter and machine cut into five triangular sectors; it is supported between the base and lid. The sample is applied at the apex of the triangle (Kawerau 1956).

Method I

Amino acids from haemoglobin of A. glabratus and P. corneus were chromatographed alongside standard amino acid solutions. Whatman No. 1 papers were used and they were irrigated once, the solvent front running to the inner edge of the ground glass flange.

Two solvents were used:

1. 250 ml. of n-butanol were shaken with 60 ml. of acetic acid and 250 ml. of water. The top layer was used as the solvent.
2. 400 g of phenol were melted by warming and 100 ml. of water added. The solvent was stored in a dark bottle, in the refrigerator, with a little sodium cyanide.

The butanol-acetic acid-water papers were dried at 75°C for 1-2 hours. The phenol papers were dried overnight, in a fume cupboard, at room temperature. Colour

was developed by dipping in 0.25% ninhydrin in acetone and heating for 15-20 minutes at 75°C.

The purpose of this series of experiments was to determine whether any differences were obvious between the patterns obtained with the two species. The standard amino acids used were 17 of the 18 that occur in horse haemoglobin (Hawk et al, 1954) - tryptophane was omitted as this is destroyed by acid hydrolysis - together with hydroxyproline.

Results

The standard amino acids formed distinct bands but many of them overlapped or were very close together and only a few could be identified from a mixture of all 18. With the butanol-acetic acid-water solvent only tyrosine and alanine separated singly. Threonine, serine and glycine formed separate bands with the phenol-water solvent.

The bands obtained with the blood samples were also very close together but it was possible to match most of the bands of the samples with one or more standard amino acids. With the butanol-acetic acid-water solvent, the faster moving substances of the two extracts were very similar, but an apparently real difference occurred in the innermost bands; the concentrations of the two extracts were approximately 10 mg./ml. A. glabratus

showed two bands, one in the position occupied by arginine and glycine and the other by lysine and cystine, which were quite distinct from one another and lightly stained. The corresponding bands in P. corneus were heavily stained and a lightly stained area occurred between them. A similar result was obtained with several samples and it appeared that there were either additional amino acids in haemoglobin of P. corneus or that the concentrations of certain amino acids were greater in P. corneus than A. glabratus.

The patterns for the two snails were similar with the phenol-water solvent. Each sample formed six bands but 12 of the standard amino acids occurred in the two outer bands which were consequently heavily stained.

Comment

Individual amino acids formed sharp bands which made the technique appear promising. The separation was poor since even the fastest moving amino acids did not reach the edge of the dish. The difference between haemoglobin of P. corneus and A. glabratus immediately made it necessary to resolve each of the amino acids in each sample and greater separation of the components was essential.

Method II

Papers were developed up to three times to increase the resolution of the bands. The solvent used was n-butanol-acetic acid-water 450:50:125. The papers were irrigated until the solvent front reached the inner edge of the lid, dried, and re-run. The quantities of amino acids from P. corneus and A. glabratus varied. The exact concentration of each sample was unknown although each extract was diluted to contain 10 mg. of haemoglobin per ml., the weight of haemoglobin being obtained before hydrolysis, so that the different solutions were probably in similar concentrations.

Results

Multiple development improved the resolution of the bands and estimations by eye suggested that the amino acid concentrations were similar. Two differences occurred between the two snails. One composite band appeared stronger in P. corneus than A. glabratus, and P. corneus also showed a strong red band which was not present in A. glabratus.

Block (1950) used this solvent to obtain separation of 10 amino acids by multiple development with ascending chromatography. Only valine and tryosine separated on the circular paper, although several others showed

incomplete separations.

Comment

Multiple development increased the rate of movement of the amino acids, but only two were resolved by the solvent used. Differences between the snail extracts were again obtained, the red colouration in extracts from P. corneus being unusual since, with this solvent, all amino acids stain a mauve or purple colour, except proline and hydroxyproline which stain yellow. Extracts from both snails varied in colour from practically colourless to light yellowish-brown. This was presumably due to hydrolysis products of the porphyrins in the haemoglobin molecule.

Greater separation, with different solvent systems, was required to permit qualitative and quantitative estimation of the amino acids. Krishnamurthy and Swaminathan (1955) separated the 16 amino acids of casein on circular paper 28 cm. in diameter. The paper was supported on a glass stand and solvent fed to the paper through a roll of filter paper cut at one end to form a brush. The whole was covered by a glass through. Five solvents were used and the papers run two or three times. Similar solvents were used with the Kawerau apparatus, but when the solvent front was kept within the edge of the dish,

separation was not satisfactory. If the solvent was allowed to run between the flanges of the base and lid, unequal movement often occurred and the chromatograms became diffuse and irregular. These limitations of the Kawerau apparatus led to the development of the final technique. ^{*}(Robinson and Targett, 1961).

c) Final Technique

The base of the Kawerau apparatus was placed in a pneumatic trough 30 cm. in diameter and 12 cm. deep. Four pieces of fine capillary tube were placed across the dish to support the paper. Solvent was placed in the Kawerau dish and in the trough. The trough was covered with a glass lid and equilibrated at 70°F.

Whatman No. 1 slotted circular papers, 26.5 cm. in diameter, were used and samples streaked 1.5 cm. from the centre of the paper. Some of the papers were previously buffered by irrigating in a standard Kawerau apparatus with buffer solution and drying at low heat. The papers were multiple developed and the amino acids detected by dipping the papers in 0.25% ninhydrin in acetone and heating for 15 minutes at 75°C.

^{*} This technique alone was developed in collaboration with Dr. D.L.H. Robinson who was investigating the amino acids of adult schistosomes.

Five solvent systems were used:

1. Phenol

Hot phenol was stirred into excess buffer solution pH 12.0 (Appendix III) and allowed to stand. The lower layer was used as the moving phase in conjunction with papers buffered at pH 12.0. Double development for 6-7 hours separated tyrosine, alanine, threonine, glycine, glutamic acid and aspartic acid (Figure 11).

2. Phenol-n.butanol-acetic acid-water

20 volumes of hot phenol were stirred into a mixture of 20 volumes of n-butanol, 8 volumes of glacial acetic acid and 40 volumes of water. After standing, the top layer was used as the solvent. Papers used with this solvent were buffered at pH 2.0 (Appendix III) before being developed three times for $5\frac{1}{2}$ - $6\frac{1}{2}$ hours. Prolonged running beyond the edge of the paper was avoided.

The solvent separated tyrosine, alanine, threonine, glutamic acid, glycine, histidine, lysine and cystine (Figure 12).

3. Cresol

Cresol (mixed isomers) was shaken with excess buffer solution pH 8.4 (Appendix III) and the lower layer used as solvent with papers buffered at pH 8.4. The papers were

TABLE III

TABLE III

Chromatographic bands separated by the five solvents

Butanol-Acetic acid-water	Phenol-Butanol-Acetic acid-water	Cresol	Phenol	Tert. Amyl alcohol-Benzyl alcohol
1. Isoleucine, Leucine.	Phenylalanine, Isoleucine, Leucine.	Phenylalanine	Arginine, Proline, Phenylalanine, Leucine, Isoleucine, Lysine	Phenylalanine
2. Phenylalanine	Tryptophane, Methionine, Valine, Proline	Proline	Methionine, Tryptophane	Leucine
3. Tryptophane	Tyrosine	Isoleucine, Leucine, Tryptophane	Valine, Histidine	Isoleucine
4. Methionine, Valine	Alanine	Methionine	Tyrosine	Methionine
5. Tyrosine	Threonine	Valine	Alanine	Tyrosine
6. Proline	Glutamic acid	Tyrosine, Histidine, Arginine	Threonine	Valine
7. Alanine	Glycine	Alanine	Glycine	Proline
8. Threonine, Glutamic acid	Serine, Arginine, Aspartic acid	Threonine	Serine, Cystine	Alanine, Threonine, Histidine
9. Serine, Glycine, Aspartic acid	Histidine	Lysine	Glutamic acid	Arginine, Cystine, Glycine, Serine, Lysine.
10. Arginine	Lysine	Glycine	Aspartic acid	Aspartic acid, Glutamic acid
11. Histidine, Lysine	Cystine	Serine		
12. Cystine		Glutamic acid, Cystine		
13.		Aspartic acid		

developed three times for 9-10 hours. Phenylalanine, proline, methionine, valine, alanine, threonine, lysine, glycine and serine separated singly (Figure 13).

4. n-Butanol-acetic acid-water

40 volumes of n-butanol and 10 volumes of glacial acetic acid were added to 50 volumes of water. The top layer was used as solvent. Unbuffered papers were developed three times for 5-7 hours and this separated tryptophane, tyrosine, proline, alanine, arginine and cystine (Figure 14).

5. Tertiary amyl alcohol-benzyl alcohol-water

50 volumes of tertiary amyl alcohol and 50 volumes of benzyl alcohol were shaken with 100 volumes of water. After standing, the top layer was used as solvent with papers buffered at pH 8.4. Krishnamurthy and Swaminathan (1955) used this solvent and stated that unbuffered papers were equally satisfactory but it was found that separations were improved by using buffered paper. The solvent separated phenylalanine, isoleucine, leucine, methionine, tyrosine, valine and proline (Figure 15). The papers were developed twice for 16 hours, the solvent being allowed to run well beyond the edge of the paper.

Table III shows the chromatographic bands separated by the five solvents. This table includes all

the amino acids which may be expected to occur in a protein hydrolysate (Meister, 1957) except glutamine and asparagine, which undergo deamination during hydrolysis to form glutamic acid and aspartic acid respectively, and cysteine which is oxidised to cystine during hydrolysis. Table IV compares the separation of individual amino acids by this method and by that of Krishnamurthy and Swaminathan (1955).

TABLE IV - Single amino acids separated by the five solvents

	Butanol/acetic acid/water		Phenol/acetic acid/water		Cresol/pH 8.4		Phenol/pH 12.0		Tert. amyl alc./benzyl alc./water
	A	B	A	B	A	B	A	B	
Phenylalanine	+	+			+	+			+
Leucine									+
Isoleucine									+
Tryptophane	+								
Methionine					+	+			+
Valine					+	+			+
Tyrosine	+	+	+	+			+		+
Proline	+	+			+				+
Alanine	+	+	+	+	+	+	+	+	
Threonine			+	+	+		+	+	
Glutamic acid			+	+			+	+	
Serine					+			+	
Glycine			+		+		+	+	
Aspartic acid					+		+	+	
Arginine	+	+				+			
Histidine			+	+		+			
Lysine			+	+	+				
Cystine	+	+	+	+					

A - Our results B - Results of Krishnamurthy & Swaminathan 1955

FIGURES 11 - 15

SEPARATION OF AMINO ACIDS BY CIRCULAR PAPER
CHROMATOGRAPHY USING FIVE SOLVENT SYSTEMS

FIGURE 11

Phenol pH 12.0

- a. Aspartic acid
- b. Glutamic acid
- c. Glycine
- d. Threonine
- e. Alanine
- f. Tyrosine

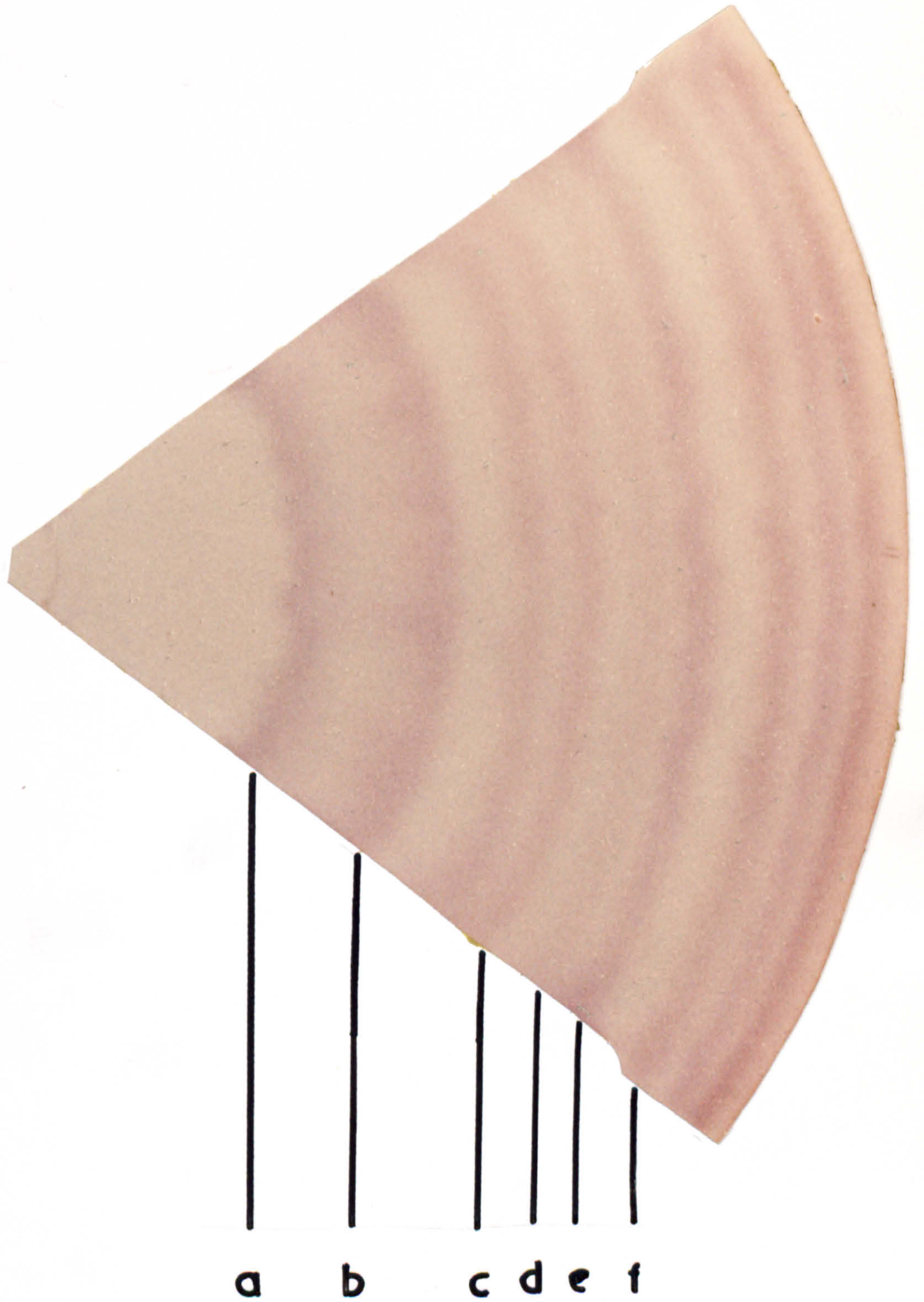
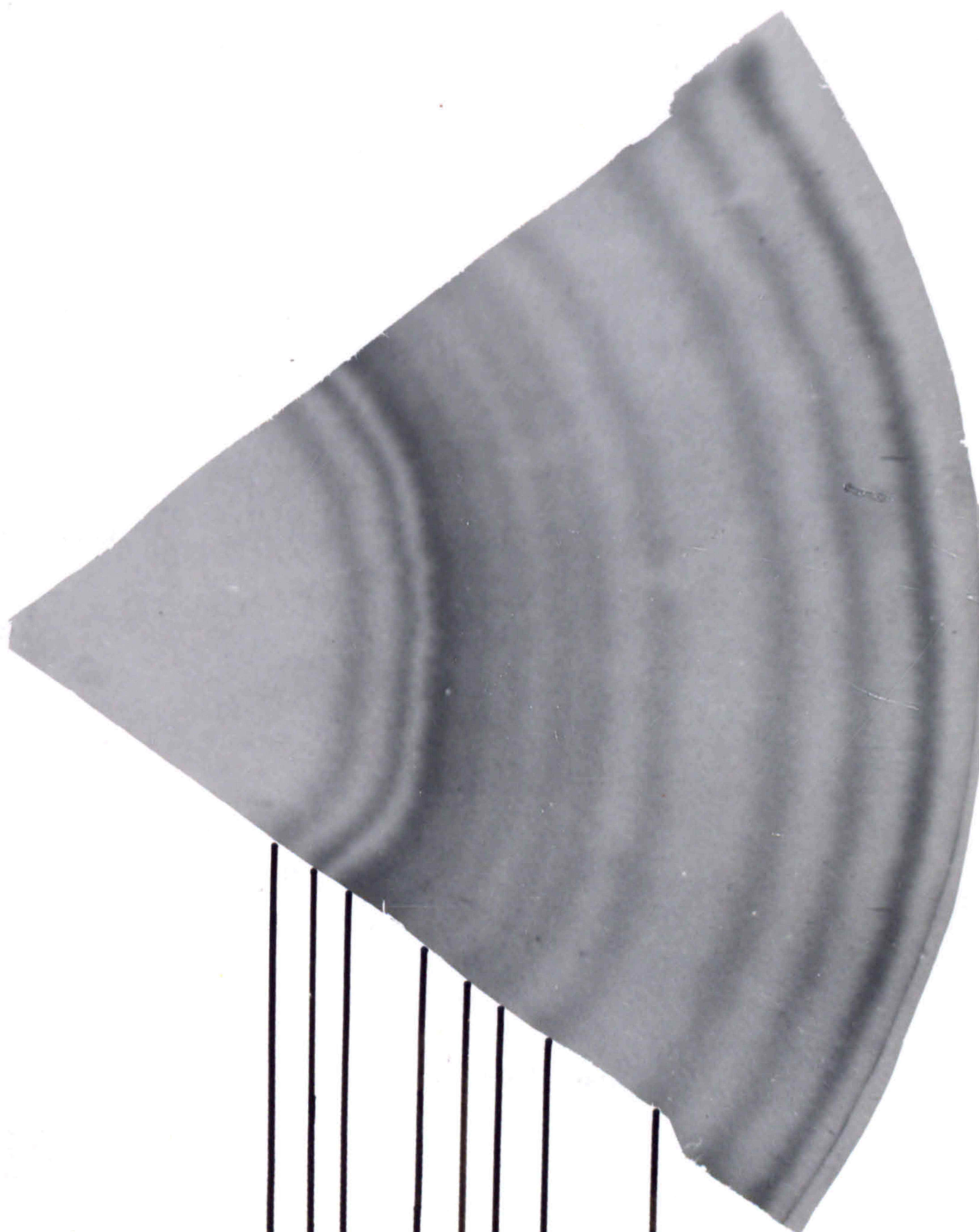
FIGURE II

FIGURE 12

271

Phenol-butanol-acetic acid-water

- a. Cystine
- b. Lysine
- c. Histidine
- d. Glycine
- e. Glutamic acid
- f. Threonine
- g. Alanine
- h. Tyrosine

FIGURE 12

abc d e f g h

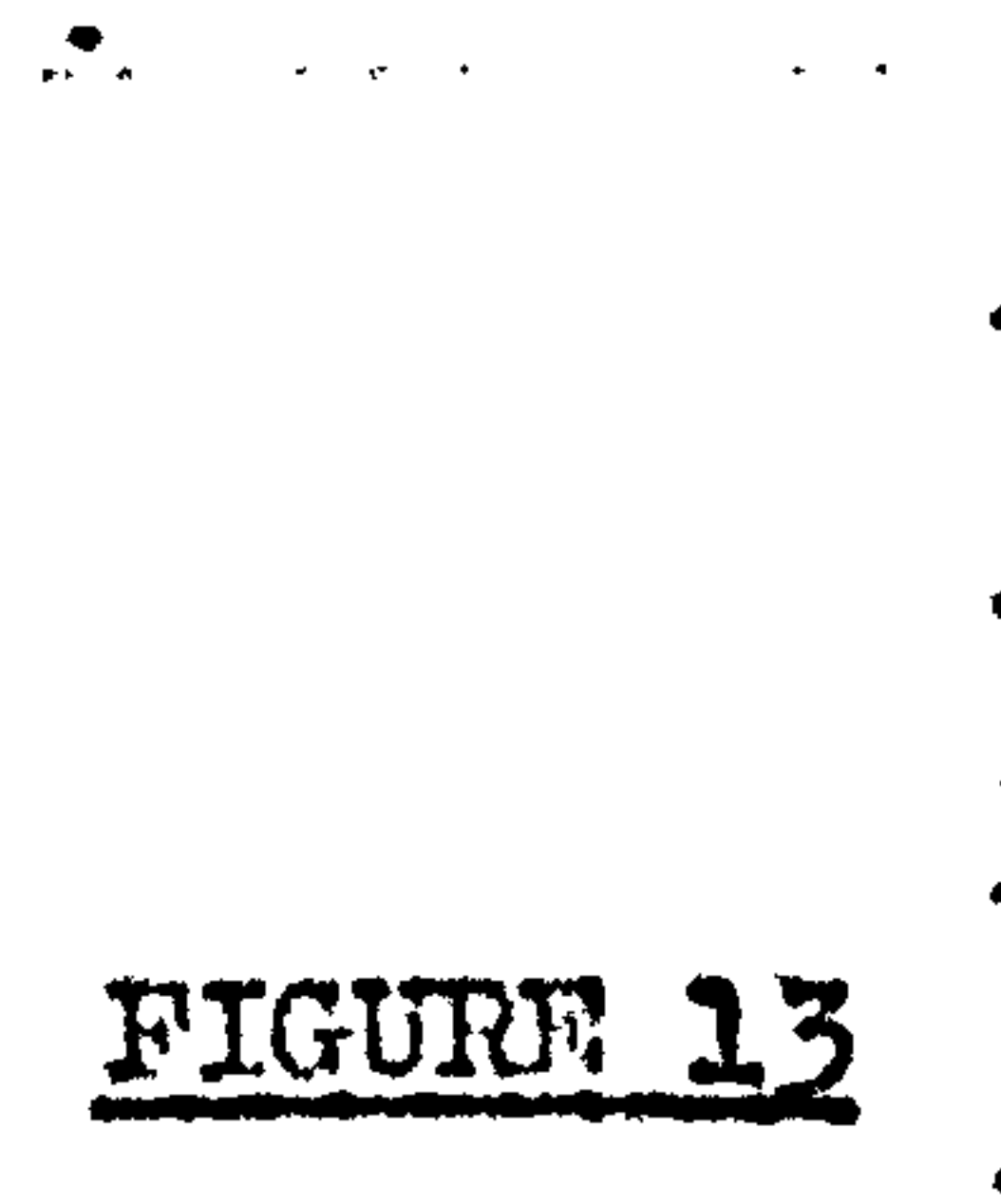


FIGURE 13

Cresol pH 8.4

- a. Aspartic acid
- b. Serine
- c. Glycine
- d. Lysine
- e. Threonine
- f. Alanine
- g. Valine
- h. Methionine
- i. Phenylalanine

FIGURE 13

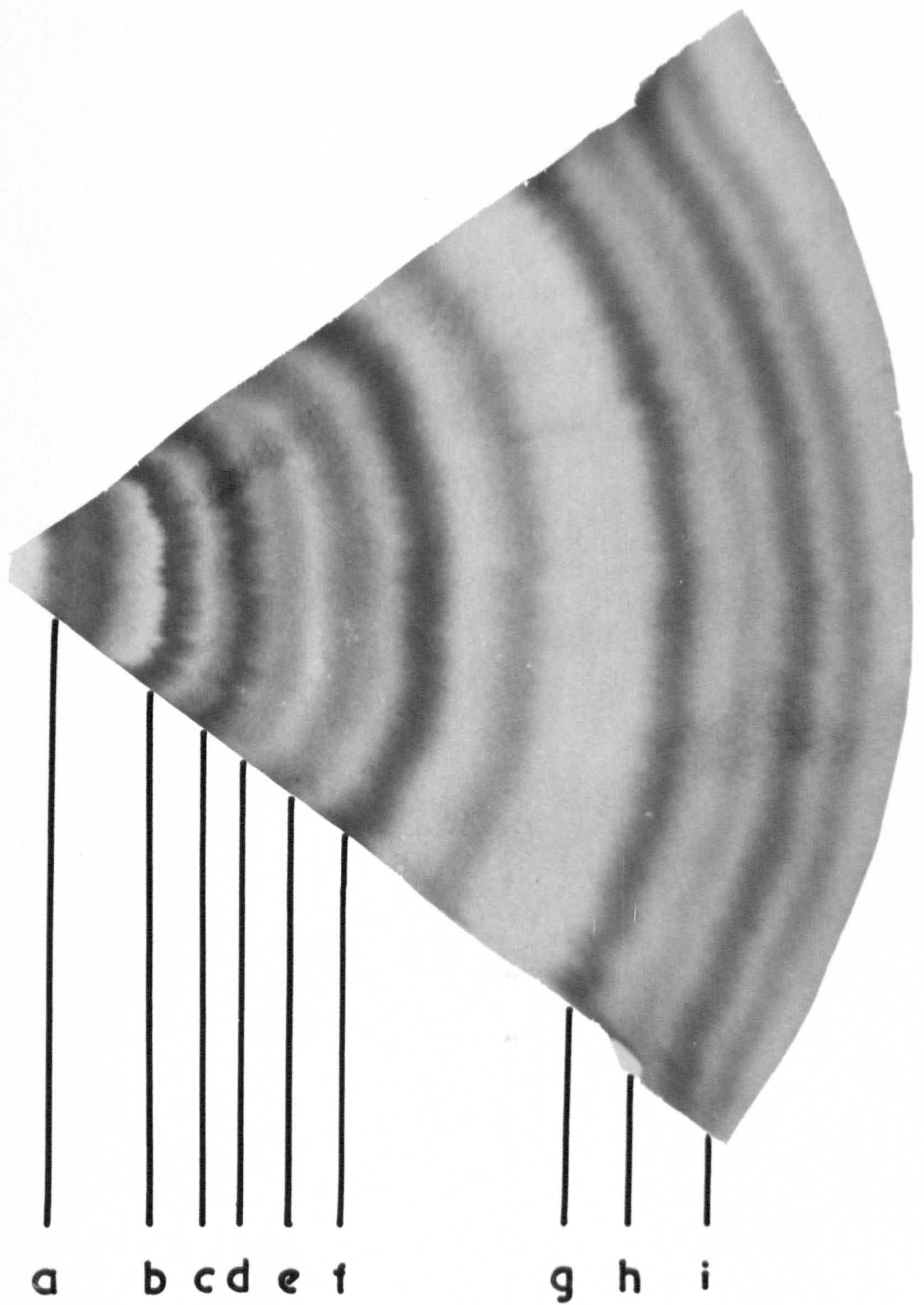


FIGURE 14

Butanol-acetic acid-water

- a. Cystine
- b. Arginine
- c. Alanine
- d. Proline (not visible in the photograph)
- e. Tyrosine
- f. Tryptophane
- g. phenylalanine (not clearly separated from
the adjacent composite band)

FIGURE 14

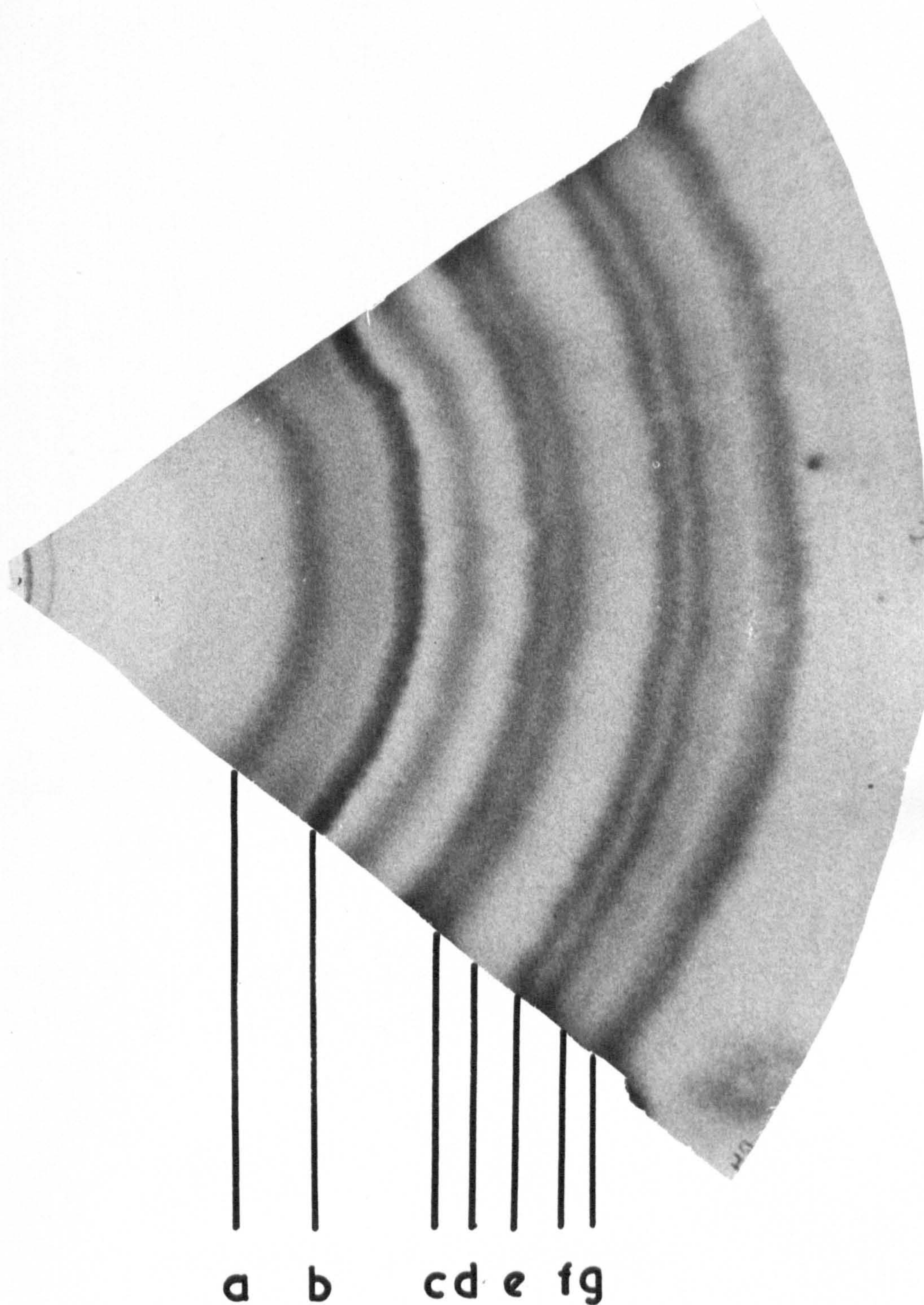
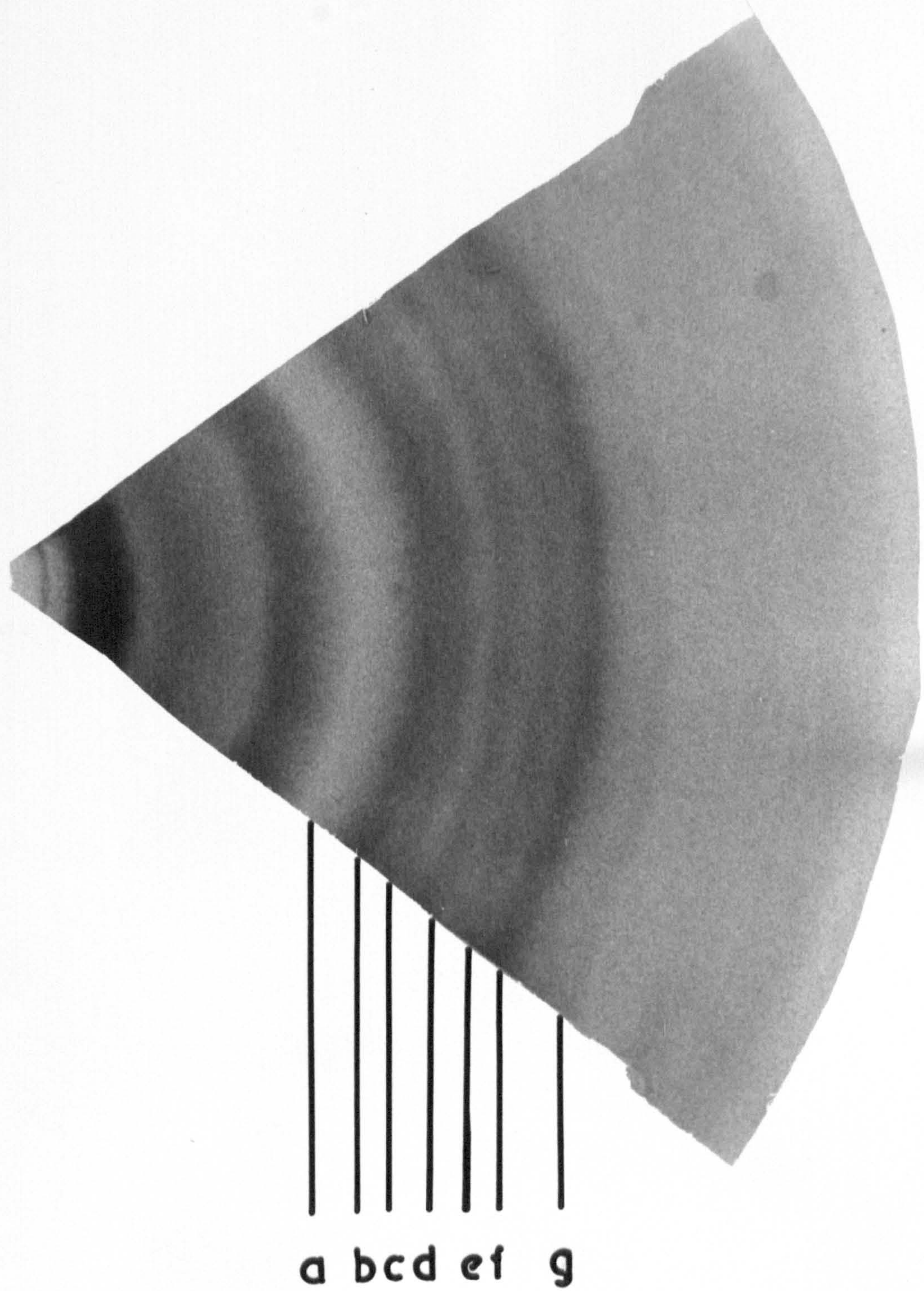


FIGURE 15

•••

Tertiary amyl alcohol-benzyl alcohol-water

- a. Proline (not visible in the photograph)
- b. Valine
- c. Tyrosine
- d. Methionine
- e. Isoleucine
- f. Leucine
- g. Phenylalanine

FIGURE 15

Quantitative Estimations

Three methods were tried for quantitative estimations. Measured amounts of the samples were run alongside varying amounts of the amino acid standards. The papers were dipped in 0.25% ninhydrin in acetone and the colour developed at 75°C for 15 minutes.

1. The stained bands were cut out and each band out into strips. The colour was eluted by shaking in a test tube for 5 minutes with 75% ethanol. Optical density measurements were made with a Unicam S.P. 500 spectrophotometer at 570 mμ. The samples were compared with known amounts of standard amino acids, the concentrations of single amino acids being proportional to the optical density.

2. Rectangular segments were cut from the sectors, cleared by immersion in paraffin oil and scanned with an Eel Transmittance Densitometer. Single amino acids in the samples were compared with known concentrations of the same amino acid treated in a similar way, the area under the curve produced by each amino acid being cut out and weighed.

3. The paper was placed over a slit of light and the transmitted light recorded through a photocell

with a microammeter. The transmittance through an unstained region of the paper was obtained and several readings made along the length of each band. These were averaged and converted to optical density. The 'area' of each band was obtained by measuring the width of the band and multiplying it by the straight line measurement between the ends of the band. The 'area' was multiplied by the optical density and the figures obtained for unknown samples compared with those obtained from various concentrations of standard amino acids.

The first method was used principally but the second technique gave similar satisfactory results. The third method gave good approximations except when amino acids were in low concentration since the technique was unsuitable for estimations on faint bands.

The total nitrogen content of each haemoglobin hydrolysate was determined by digesting measured amounts with sulphuric acid, the ammonia formed being determined quantitatively, with a spectrophotometer, using indane-trione hydrate (Jacobs 1959) to produce the colour.

Preparation of Samples

In previous experiments the final solution of blood protein amino acids often contained a coloured contaminant

probably produced by hydrolysis products of the prosthetic portion of the molecule. A polystyrene resin was obtained which removed it, but the behaviour of the coloured material was unusual. Some of the colour passed through the column but the remainder was held by the resin and was eluted at the same time as the amino acids with ammonia solution. When the ammoniacal solution was evaporated to dryness and isopropanol added, the amino acids dissolved but the pigment was generally insoluble, whereas it readily dissolved in isopropanol before being passed through the resin. The resin used was Dowex 50, 100-200 mesh, but the cross linkage was unknown. It seemed rather crude when compared with other resins but retained its property after being thoroughly washed and reclaimed (Appendix IV). Other resins tried were less efficient, some of the colour remaining even after passing the sample through a short column two or three times.

d) Results

(1) Qualitative Estimation of Amino Acids of Snail Blood Proteins

The following amino acids were identified in haemoglobin extracts of A. glabratus, P. corneus, B.(P). globosus and B.(P). nasutus, and in haemocyanin from L. stagnalis:

Isoleucine	Tyrosine	Serine	Lysine
Leucine	Proline	Glycine	Cystine
Phenylalanine	Alanine	Aspartic acid	
Methionine	Threonine	Arginine	
Valine	Glutamic acid	Histidine	

In addition, one unidentified amino acid occurred in A. glabratus, P. corneus, B.(P). nasutus and L. stagnalis. This band did not correspond to any of the 18 amino acid standards or to any of 11 other amino acids which were run against the samples. Tryptophane was not detected in the alkaline hydrolysates.

Occasionally, two or three faint bands occurred near the point of application with phenol-butanol-acetic solvent. Ornithine behaved in a similar way and since these bands appeared only irregularly, it was assumed that they were due to ornithine formed by the breakdown of arginine during acid hydrolysis (Meister, 1957).

Methionine was present in low concentration in all the extracts and occasionally samples were obtained in which this amino acid did not show.

Comment

No qualitative differences were obtained between the different haemoglobins and the haemocyanin, except the absence from the haemoglobin of B.(P). globosus of the unidentified amino acid. This band was very lightly

stained and showed only when relatively large amounts of material were used. There was less material available from B.(P). globosus than from the other snails so that the concentration on the paper may have been too low to show this substance.

Comparisons between samples of similar total concentrations suggested that the amounts of single amino acids present in the different snail haemoglobins showed only very slight differences when examined by eye. Thus, methionine was slightly more clear in P. corneus, although this band varied, as already mentioned, in different samples. Glutamic acid seemed stronger in haemoglobin from B.(P). nasutus.

The haemocyanin extract showed a stronger proline, slightly more tyrosine, methionine and phenylalanine, but less aspartic acid.

A number of extracts and standard amino acids used in one series of experiments had been stored several months before use. The samples and standards showed additional red or reddish-brown bands which were not normally obtained. When the standard amino acids were examined singly, lysine, particularly, formed up to four bands, one or two of which were red in colour. Serine also split forming a faint red band. It is possible

TABLE V

TABLE V

Comparative Amounts of Selected Amino Acids
in Haemoglobin from Three Snail Species.

Amino Acid	<u>Species</u>		
	<u>A. glabratus</u>	<u>P. corneus</u>	<u>B. (P). nasutus</u>
Phenylalanine	3.37	3.48	3.02
Isoleucine	3.87	4.61	3.84
Leucine	5.89	6.54	5.95
Alanine	11.70	9.75	9.00
Threonine	3.92	3.96	3.12
Glycine	7.67	7.72	7.10
Glutamic acid	6.26	8.08	9.30
Aspartic acid	10.63	13.90	8.87
Lysine	12.82	16.40	13.20
Valine	6.43	4.64	5.42
Tyrosine	2.73	2.82	1.57

The figures represent the amino acid nitrogen as a percentage of the total nitrogen in an acid hydrolysate.

that the red colour noted in earlier experiments (page 82) may have been due to the breakdown of one or more amino acids.

(ii) Quantitative Estimations of Amino Acids of Snail Blood Proteins

Estimations were made of selected amino acids from haemoglobin samples of A. glabratus, P. corneus and B.(P). nasutus. All the amino acids present in the samples were not estimated for a number of reasons.

Some were present in varying amounts either through partial destruction during hydrolysis, as occurred with serine, or because the amino acid split into two or more bands, as occurred sometimes with cystine. Other amino acids, while always identifiable, were not always sufficiently sharply separated from an adjacent band to be cut out accurately. This occurred with proline, histidine and arginine. Most of the amino acids which were selected for estimations could be separated singly with more than one solvent.

Results

Table V shows the comparative amounts of eleven amino acids in haemoglobin from three snail species. The figures represent amino acid nitrogen as a percentage

of the total nitrogen in each acid hydrolysate. This method was more accurate than measuring the total amino acids as a percentage of total protein, since this would have involved conversion of the figures obtained for total nitrogen in each sample into values for total protein, a conversion which is at best only an approximation, and in this instance would have been applied to a conjugated molecule, part of which had been removed during hydrolysis. Since the structure of the snail haemoglobin molecule was unknown, particularly the relationship between the protein and non-protein portions of the molecule, it would have been unwise to have applied the conversion factor (nitrogen value $\times 6.25$) used for simple proteins.

Comment

Comparisons of the amino acids from haemoglobins of the three species show that, with many of the amino acids, differences in concentration occur but the levels of each substance in the different samples are of the same order. The differences between concentrations of some other amino acids are more significant although again the pattern is similar in the three snails. Thus, while A. glabratus has a greater amount of alanine than the other snails, and P. corneus a higher level of lysine, both these substances occur in high concentration in the

three species. The most marked differences appear to be between the concentrations of aspartic acid and glutamic acid. If the snail haemoglobin is composed of units with a molecular weight of 34,500 (Svedberg, 1933), the differences can be related very approximately to the number of molecules of these amino acids in the haemoglobin unit. The figures for glutamic acid nitrogen in Table V are: A. glabratus, 6.26; P. corneus, 8.08; and B.(P) nasutus, 9.3 per cent of total protein nitrogen. In terms of molecules, there are about 25 molecules of glutamic acid in one unit of A. glabratus globin, 32 molecules in the P. corneus globin and 37 molecules in the B.(P). nasutus globin. The aspartic acid figures are: A. glabratus, 10.63 (42 molecules); P. corneus, 13.9 (55 molecules), and B.(P). nasutus, 8.87 (35 molecules).

The amino acid composition of other invertebrate haemoglobins has been determined for relatively few species and comparison is, therefore, difficult. Roche and co-workers (1934a, 1934b, 1937) determined the concentration of certain amino acids in annelid haemoglobin pigments. Where comparison of these with snail haemoglobin was possible, the most marked difference was the low level of lysine in the annelid pigments as

opposed to a high concentration in the snail haemoglobins.

There are also numerous differences in concentration of snail amino acids when compared with mammalian haemoglobins (Lemberg and Legge 1949).

The results suggest that haemoglobins of snail species have developed in response to general common requirements. While the non-protein portions of the molecules are probably the same in all planorbid snails, the amino acid composition of the globins varies to a greater or lesser degree from one snail group to another. Whether there are, in fact, species differences, or whether there are patterns common to larger groups of snails, remains to be determined by examination of snails more closely related taxonomically. It has been suggested (Svedberg, 1933) that the invertebrate haemoglobins of high molecular weight are built up from units of similar molecular weight to the units composing the mammalian haemoglobins. Variation in amino acid composition of the haemoglobins of snails and annelids (Lemberg and Legge, 1949) are greater than the differences between mammalian types. It may be that, whereas mammalian haemoglobins were developed from a common haemoglobin unit, the invertebrate pigments were developed from a simpler protein structure in response

to a common set of conditions, and although as a result their composition is broadly similar, it varies more than that of mammalian pigments.

2. Free Amino Acids of Snail Blood

a) Preparation of Samples

Blood was obtained from snails by the technique previously described and absolute alcohol added to the pooled blood samples to give a final solution of 80% alcohol. In general, 2 ml. of blood were used for each extract. The solution was centrifuged to remove the precipitated protein and the supernatant, containing the free amino acids, added to three volumes of chloroform (Awapara, 1948) and left to stand for 6-12 hours. The upper aqueous layer was removed and the chloroform layer washed with 0.01N.HCl in order to obtain maximum recovery of the amino acids. The mixture was partitioned again and the upper layer removed and added to the first aqueous solution. This often contained a small amount of colour which was removed by passing the solution through the Dowex-50 resin column and eluting the amino acids with 10% ammonia solution. The ammoniacal solution was evaporated to dryness and the residue dissolved in a small amount of 10% isopropanol, generally 0.2 ml.

Extracts were prepared from A. glabratus, P. corneus and L. stagnalis which had been bred and maintained in the laboratory. In addition, blood was obtained from

TABLE VI

TABLE VI

Free Amino Acids of Snail Blood

	A. glabratus	P. corneus lab. bred	L. stagnalis lab. bred	P. corneus Frogmore	L. stagnalis Frogmore
Isoleucine	} +	} +	} +	} +	} +
Leucine	} +	} +	} +	} +	} +
Phenylalanine	+	++	+	-	-
Tryptophane	-	-	-	-	-
Methionine	-	-	-	-	-
Valine	++	++	++	++	++
Tyrosine	+	++	++	++	+
Proline	-	-	-	-	-?
Alanine	++	++	++	++	++
Threonine	++	++	++	++	++
Glutamic acid	++	++	++	++	++
Serine	++	+	++	++	++
Glycine	++	++	++	++	++
Aspartic acid	++	++	++	++	+
Arginine	-	-	-	-	-
Histidine	-	-	-	-	-
Lysine	+	+	++	+	+
Cystine	++	++	+	++	+
Unidentified	-	-	1(+)	1(+)	-

++ Identified in every sample

+ Identified in some of the samples

- Not detected

P. corneus and L. stagnalis which were collected from a river at Frogmore, Hertfordshire. The latter were examined for trematode infections immediately after collection and blood was taken only from snails which did not emit any cercariae.

b) Method

The amino acids were determined using the circular chromatography method already described. Because of the low concentrations of free amino acids it was often necessary to apply a large amount of fluid, up to 0.15 ml., the amount of solution being limited by the strength of the paper.

c) Results

Table VI shows the amino acids detected in the five extracts. The amount of free amino acids was low in each case and detection of single amino acids depended in part on the amount of material applied to the paper. Despite this, some amino acids were identified from every sample, and these are indicated in the table. Others occurred in some but not all of the sample while a few were identified on very few occasions. Thus phenylalanine in A. glabratus and tyrosine in A. glabratus and L. stagnalis (Frogmore) were each identified only once. Proline was not

detected in any of the extracts but in one sample from L. stagnalis (Frogmore) there was a suggestion that proline was present although it could not be positively identified.

It was not possible to separate iso-leucine and leucine, even with the benzyl alcohol-tert. amyl alcohol solvent, as the concentration was too low. The unidentified band which occurred in L. stagnalis (Laboratory-bred) and P. corneus (Frogmore) was very faint and was the same substance which showed in the pigment proteins (vid. sup.).

Comment

The amount of free amino acid in snail blood is very small and the amounts of some amino acids varied with different samples, but no quantitative estimations were made. This was more noticeable with L. stagnalis and P. corneus since large specimens were available and relatively few snails were necessary to obtain 2 ml. of blood whereas 20-30 A. glabratus were generally required. The amount of free amino acid seemed to be lower, however, in A. glabratus kept in poor conditions with little or no light and no aeration.

Comparisons in different samples from each species of the amino acids which occurred regularly in the five extracts showed little variation in the amounts of valine,

alanine and glycine. They were relatively strong in the five groups of snails. Threonine and glutamic acid were present in similar concentrations, with little variation from sample to sample, in the Frogmore snails, but each laboratory-bred species showed considerable variation in the amounts of these amino acids obtained from different samples.

There was a distinct difference between the phenylalanine content of laboratory-reared A. glabratus and P. corneus. This amino acid was always clearly present in P. corneus but occurred only once, and in very low concentration, in A. glabratus. Tyrosine, also, was identified only once in A. glabratus, again in low concentration, but was always definitely present in P. corneus. The amino acids which did not occur regularly were, when present, generally in very low concentration.

The results show that quantitative differences probably occur between the snail species. The most marked differences between the laboratory-reared snails are the low levels of phenylalanine and tyrosine which occur in A. glabratus. The wild specimens of P. corneus and L. stagnalis showed similar amino acids to the laboratory stock except that phenylalanine was apparently absent.

The most significant point from the results, however, was the apparent absence from all the snails of methionine, proline, arginine and histidine, which would be expected in protein tissues. As they occur in other snail tissues they are probably obtained by transamination or by rapid absorption from the blood leaving the amino acid level too low to be detected by this method.

General Comment

These experiments have shown that the haemoglobins of different snail species are, in general, similar in structure and behaviour. Electrophoresis under varying conditions produced similar results in all the species of snails examined and the absorption spectra suggested that the non-protein portions of the molecules were the same.

The differences in amino acid composition may produce some variations in the properties of the different molecules. It has already been shown, that in snails containing haemocyanin, the pigment protein has an important part in the maintenance of the internal environment. Since haemoglobin is generally the only protein present in the snail blood it will presumably also play an important role in the maintenance of osmotic pressure and the levels of inorganic constituents. The large invertebrate haemoglobin molecules are considered, as stated earlier, to be formed of many small units (Svedborg 1933, Lemberg and Legge, 1949) which are united, not in a long chain but in a more complex manner. Inorganic salts combine with this molecule (Lemberg and Legge 1949) and the differences detected in these experiments between the three snail haemoglobins may affect this protein-salt balance, producing different levels of inorganic constituents in the blood. The snails

examined were not closely related species taxonomically; such species should be compared to determine whether haemoglobins are indeed species-specific.

The other blood constituents examined showed no definite features which might characterise one group of snails when compared with other groups. The non-pigment blood proteins occur only sporadically in all the snails and would not, therefore, influence the maintenance of the general internal environment.

The free amino acids are present in low concentration and qualitatively similar in all the snails with no indication of marked differences between susceptible and non-susceptible stock.

The absence, from the blood, of amino acids which were present in the haemoglobin molecule and would be expected in other proteins led to an investigation of the free amino acids of other snail tissues.

PART II

PROTEIN AND NON-PROTEIN AMINO ACIDS
OF SNAIL TISSUES OTHER THAN BLOOD

Protein and non-Protein Amino Acids
of Snail Tissues other than Blood

Introduction

The development of schistosome sporocysts involves drastic changes and great increases in size. A considerable build-up of protein material occurs from amino acids drawn from the snail. The requirements of the parasite might come from bound or free amino acids of the host but since the primary sporocyst, at least, settles and develops in many different regions of the snail, preliminary examinations must be made of the free amino acids. The level of free amino acids in the blood is very low in all the snails examined, with some amino acids which are commonly present in protein material either absent, or present in concentrations too low to be detected. This led to an investigation of the amino acids in other tissues of the snail. Comparative studies were made of the free amino acids of the tissues of the anterior region of the snail, the amino acids most readily available to the primary sporocysts. Bound and free amino acids of the digestive gland and ovotestes were also examined to compare the amino acids utilised by different snail species and to determine the amino acids available to the

secondary sporocysts.

a) Preparation of samples

Three extracts were prepared from tissues of snails which had been bled. After withdrawal of blood the snails were removed from the shells and the digestive gland and ovotestes dissected out and placed in 80% alcohol. The gut was removed completely from the anterior tissue remaining which was placed in 80% alcohol.

The digestive glands and ovotestes were homogenised at 14,000 r.p.m. for 5 minutes (L.S.E. homogeniser fitted with a 10 ml. Vortex flask), then centrifuged and the supernatant removed. The residue was washed with 80% alcohol and re-spun; this was repeated twice more and the combined alcoholic solutions added to 3 volumes of chloroform (Awapara 1948) and left to stand for 6-12 hours. The upper aqueous layer was removed and the chloroform washed with 0.01N.HCl, in a similar way to that used for recovery of free amino acids of the blood. The solution obtained generally contained a considerable amount of colour and was passed through a Dowex-50 resin column and the amino acids recovered by the method already described (vid. sup.). It was often necessary to pass the solution through the resin a second time. The amino acids were dissolved finally in a small amount of 10% isopropanol

generally 0.5 ml.

The residue after homogenising, containing protein, was hydrolysed with 6N.HCl for 24 hours at 120°C. The acid solution was centrifuged and the supernatant removed. This was generally dark brown in colour and it was necessary to pass the solution two or three times through the resin column in order to obtain a colourless solution. The amino acids were finally dissolved in 10% iso-propanol.

The remaining anterior tissues of the snail were treated in a similar way but only the free amino acid extract was prepared. The three extracts were designated as follows:-

1. Digestive gland and ovotestes, hydrolysed.
2. Digestive gland and ovotestes, free.
3. Anterior tissues, free.

Extracts of hydrolysed digestive glands and ovotestes were prepared from laboratory-bred A. glabratus, P. corneus and L. stagnalis and free amino acid extracts, of the digestive gland and ovotestes and of the anterior tissues, from these snails and from P. corneus and L. stagnalis from Frogmore, Hertfordshire.

TABLE VII

TABLE VII

Free Amino Acids of the Digestive Gland
and Ovotestes

	A. glabratus	P. corneus Lab. bred	L. stagnalis Lab. bred	P. corneus Frogmore	L. stagnalis Frogmore
Isoleucine	++	++	+	} ++	} ++
Leucine	++	++	+	} ++	} ++
Phenylalanine	++	++	+	++	++
Tryptophane	-	-	-	-	-
Methionine	++	++	+	++	++
Valine	++	++	++	++	++
Tyrosine	++	++	++	++	++
Proline	++	++	++	++	++
Alanine	++	++	++	++	++
Threonine	++	++	++	++	++
Glutamic acid	++	++	++	++	++
Serine	++	+	+	++	++
Glycine	++	++	++	++	++
Aspartic acid	++	++	++	++	++
Arginine	+	+	+	+	+
Histidine	+	+	+	+	+
Lysine	++	++	+	++	++
Cystine	++	++	+	++	++
Unidentified	1	1	1		1

++ Identified in all samples
+ Identified in most samples
- Not detected

b) Results

1. Protein amino acids of the digestive gland and ovotestes

The following amino acids were identified in the three snail species:

Isoleucine	Glutamic acid
Leucine	Serine
Phenylalanine	Glycine
Methionine	Aspartic acid
Valine	Arginine
Tyrosine	Histidine
Proline	Lysine
Alanine	Cystine
Threonine	

These were in fact the same amino acids detected in the blood pigments except that the unidentified band which occurred in the pigments did not show in these extracts.

2. Non-protein amino acids of the digestive gland and ovotestes.

Table VII shows the amino acids detected in the five extracts. Distinction is again made between the amino acids which were identified in every sample and those which, in this case, showed in most but not all of the chromatograms. The unidentified substance in four of the extracts was the same one obtained in the pigment experiments.

TABLE VIII

TABLE VIII

Free Amino Acids of Anterior
Tissues

	A. glabratus	P. corneus Lab. bred	L. stagnalis Lab. bred	P. corneus Frogmore	L. stagnalis Frogmore
Isoleucine	++	} ++	} ++	++	++
Leucine	++			++	++
Phenylalanine	++	++	+	+	+
Tryptophane	-	-	-	-	-
Methionine	++	-	-	-	-
Valine	++	++	++	++	++
Tyrosine	++	++	++	++	++
Proline	++	++	++	++	++
Alanine	++	++	++	++	++
Threonine	++	++	++	++	++
Glutamic acid	++	++	++	++	++
Serine	++	+	++	++	++
Glycine	++	++	++	++	++
Aspartic acid	++	++	++	++	++
Arginine	+	+	-	+	+
Histidine	+	+	-	+	+
Lysine	++	++	++	++	++
Cystine	++	++	++	++	++
Unidentified		1	1	1	

++ Identified in all samples
 + Identified in most samples
 - Not detected

3. Non-protein amino acids of anterior tissues

Table VIII shows the amino acids identified in these tissue extracts. The unidentified band is the one obtained previously with haemoglobin samples.

a) Comment

Hydrolysates of the digestive glands and ovotestes showed that the three species utilise the same amino acids. Thus the qualitative requirements of the different species are the same and examination of the free amino acids of these organs showed all the amino acids required for the protein build-up. The total free amino acid content of the digestive gland and ovotestes was high in all the snails and although variations in the amounts of single amino acids occurred within and between species, no important species differences were obtained. Comparisons between snails from different habitats, namely snails bred in the laboratory and those obtained wild, seem to indicate that a species selects only the amino acids it requires regardless of the food material. This can only be assumed, however, since the amino acid content of the food material available to the snails was unknown and, indeed, the gut of the snails was removed so that only those amino acids actively used by the snail and not any which might be present in the food but pass unused through the animal, would be recorded.

The free amino acids of the remaining tissues of the snail showed interesting results. In the three species, amino acids which were not detected in the blood were present in the tissues. Thus arginine, histidine and proline occurred in the tissues of four groups of snails but did not show in their blood. These amino acids are available in the digestive gland and presumably protein digestion and absorption takes place in the region of the digestive gland and the blood system transports the amino acids to the other tissues. The absence of the amino acids in the blood may have been due, as already suggested, to a rapid uptake of the substances in the tissues with a consequent blood level which was too low to detect.

Two of these amino acids, arginine and histidine, did not occur in the anterior tissues of laboratory bred L. stagnalis but showed generally in the other laboratory bred species and in L. stagnalis from Frogmore. The most marked difference between the species, however, was the absence of methionine from anterior tissues of all the snails except A. glabratus where it showed in every sample. This amino acid was present in free and bound amino acid extracts of the digestive glands of all the snails examined.

The results show that, by and large, the non-

intermediate hosts of schistosomes, P. corneus and L. stagnalis, have amounts of free amino acids in their anterior tissues and digestive glands which are comparable with those of A. glabratus. Two exceptions are the apparent absence from the anterior tissues of L. stagnalis of arginine and histidine and the absence of methionine from tissues of this snail and P. corneus. It has already been shown that miracidia from *S. mansoni* will penetrate P. corneus (page 10), and the ability of the parasite to develop in a snail probably depends to a considerable extent on the degree of physiological compatibility between the parasite and the host; failure to develop being due to the internal environment of the snail proving unsuitable for the parasite rather than to a direct attack on the invader by phagocytes of the snail. Thus, it may be that differences in, for example, the amino acids available to the parasite may decide whether a snail can harbour a particular strain or species of parasite.

These studies led to an examination of the amino acids required by the schistosome parasite and the effect of infection on the free amino acids of the snail intermediate host.

PART III

AMINO ACIDS FROM TISSUES OF INFECTED

A. GLABRATUS AND FROM CERCARIAE OF

S. MANSONI

Amino Acids from Tissues of Infected
A. Glabratus and from Cercariae of
S. mansoni

Introduction

Infection of snail by schistosome parasites often causes the death of the snail during development of the parasite, or, more frequently, when the infection is mature. There is obviously a considerable drain on the reserves of the snail yet little information is available about the effects of the schistosome on its host. Von Brand and Files (1947) found that infection of A. glabratus with S. mansoni produced no change in the fat content or oxygen consumption of the snail, but the polysaccharide content was reduced. Histochemical examination showed that sporocysts were poor in glycogen and the glycogen content of the cercariae was variable.

The present study was concerned with determination of the amino acids of the parasite and the effect of infection on the free amino acid content of the snails. An attempt was made to examine primary and secondary sporocysts of S. mansoni with associated studies of infected A. glabratus. Removal of primary sporocysts proved impracticable and it was difficult to obtain secondary sporocysts completely free from digestive gland. The study was limited, therefore, to an examination of

the bound amino acids of cercariae - there are no free amino acids present - and the free amino acids of the anterior tissues and blood of snails having mature infections. The digestive gland and ovotestes, containing mature secondary sporocysts, were also examined for free amino acids although it was initially intended to examine the snail and parasite tissues separately.

a) Preparation of samples

Extracts from infected A. glabratus were prepared in a similar way to those of uninfected snails. Blood was obtained from the snail which was then removed from the shell and the digestive gland and ovotestes, and the remaining tissues, separated from the gut.

Cercariae, liberated from snails, were concentrated in a small amount of water and absolute alcohol was added to make an 80% alcohol solution which was centrifuged at approximately 1500 r.p.m. The cercariae, generally in batches of about 100,000, were homogenised by grinding in a hard glass test tube, centrifuged, and the supernatant removed. The residue was hydrolysed for 24 hours with 6N.HCl at 120°C in a sealed ampoule. The acid solution was centrifuged and the supernatant removed and passed through a Dowex-50 resin column. The amino acids were eluted with 10% ammonia solution, evaporated to dryness and dissolved

TABLE IX

TABLE IX

Free Amino Acids from infected <u>A. glabratus</u>		Bound Amino Acids from <u>S. mansoni</u> <u>cercariae</u>		
	Blood	Anterior Tissues	Digestive gland Ovotestes Sporocysts	Cercariae
Isoleucine	} +	+	+	+
Leucine		+	+	+
Phenylalanine		+	+	+
Tryptophane	-	-	-	-
Methionine	-	-	-	+
Valine	+	+	+	+
Tyrosine	+	+	+	+
Proline	-	+	+	+
Alanine	+	+	+	+
Threonine	+	+	+	+
Glutamic acid	+	+	+	+
Serine	+	+	+	+
Glycine	+	+	+	+
Aspartic acid	+	+	+	+
Arginine	-	+	+	+
Histidine	-	-	-	+
Lysine	+	+	+	+
Cystine	-	+	+	+

in 10% isopropanol.

b) Results

Table IX shows the free amino acids detected in the blood, anterior tissues of infected A. glabratus, digestive glands and ovotestes containing mature secondary sporocysts, and the bound amino acids from cercariae of S. mansoni.

c) Comment

The quantity of material available from infected snails was much less than that from uninfected specimens, but rough comparisons, from batches of extracts from similar numbers of snails, suggested that the free amino acid content of infected animals was less than that of uninfected snails.

The amino acid level of the blood was very low and two amino acids, phenylalanine and cystine, which were present in uninfected specimens, did not show in these extracts. While direct quantitative comparisons between infected and uninfected snails were not possible, there seemed to be an overall reduction in the amount of free amino acids in the blood, which may have resulted in the levels of phenylalanine and cystine becoming too low to be detected.

The overall amounts of free amino acids in the

tissue and digestive gland extracts were also somewhat reduced and two amino acids, histidine and methionine, which were present in uninfected specimens, did not show. The level of histidine in the tissues and digestive gland of uninfected specimens was low - it was not detected in some samples - but methionine occurred regularly in both extracts. The absence or reduction in amount of methionine in infected snails is particularly interesting since, while this amino acid was present in digestive gland extracts of all the snail species examined (Table VII) it was detected only in tissue extracts from A. glabratus (Table VIII). Methionine was present in the protein of the cercariae, and this, together with the reduction in the amount of the amino acid in the snail, suggests that the parasite obtains methionine directly from the snail.

It is probable that the developing primary sporocyst draws methionine directly from the snail, but of the species examined only A. glabratus, the intermediate host, showed a measurable amount of methionine in the region of the snail where the primary sporocyst would normally settle. Thus, the level of methionine may be a limiting factor in the development of strains of S. mansoni in different species of snail.

The level of non-protein methionine was shown to

be important to the successful development of some plasmodia parasites. The main source of their amino acids is the breakdown of haemoglobin but P. knowlesi requires additional methionine which it obtains from the surrounding plasma (McKee and Geiman 1948 in Lwoff 1951). In vivo studies with monkeys showed that the hosts required methionine in their diet for growth and multiplication of the plasmodia, and injection of methionine into fasting infected monkeys produced massive infections. L-methionine could be used as a substitute for mixtures of amino acids in media suitable for cultures of P. knowlesi. Mammalian haemoglobin contains only 1% methionine and it was thought that the plasmodia had to draw on the surrounding plasma since most proteins contain 3-4% of this amino acid.

The developing sporocysts of schistosome parasites may require a similar level of methionine and, although the source of the total amino acids utilised by the parasite is not known for certain at present, it appears that much must be derived from free amino acids, and that development may be governed, in part, by the amount of methionine available. The secondary sporocysts of schistosomes are concerned solely with the rapid development and production of cercariae. It is probable, therefore, that there will be a rapid build-up of material

obtained from the snail, and amino acids will be quickly built into protein units so that the level of free amino acid in the sporocyst will always be very low. This is further suggested by the results obtained with extracts from digestive glands and ovotestes containing mature sporocysts; the amount of free amino acid seemed to be low and two amino acids, which are normally present, were not detected.

Further quantitative studies of amino acids from infected and uninfected snails, with associated studies, if possible, of bound and free amino acids of primary and secondary sporocysts, may determine the extent of the demand of the parasite on the amino acids of the snail, and the levels of different amino acids necessary for successful development of the parasite. Such work may show whether the quantitative level of one or more amino acids is a limiting factor in the ability of a snail species to support a particular parasite.

PART IV

SUMMARY AND CONCLUSIONS

FUTURE WORK

Summary and Conclusions

This investigation formed part of a study to determine the variability in the physiology of different snail species which might influence the susceptibility of the snails to infection by schistosomes.

Most of the important schistosome species develop in intermediate hosts from the small group of snails which contain a haemoglobin blood pigment. Within this group, however, there are considerable variations in susceptibility to infection with different schistosome parasites.

Comparative studies of the blood and respiratory pigments were made, to determine the variability between clearly separated snail species.

The absorption spectra of haemoglobins from A. glabratus and P. corneus and from B.(P). globosus and B.(B). tropicus angolensis were established. There was no difference between the spectra from snails which transmit S. mansoni or S. haematobium and the spectra from related insusceptible species. Snail haemoglobins produce absorption spectra which closely resemble those from mammalian haemoglobins; however, the peak at 330-340 mμ. in mammalian pigments is formed at 346-348 mμ. in snail pigments. In the ultra-violet region, snail

blood sometimes produced up to six additional peaks and this gave an indication that proteins other than the respiratory pigments could be expected.

These proteins were demonstrated by electrophoresis of snail blood on cellulose acetate membrane strips. They were common in B.(P) nasutus but rare in nine other species, two of which, L. stagnalis and O. hupensis, do not contain a haemoglobin pigment. They appeared only in medium-sized snails and no correlation could be established with egg-laying, desiccation, starvation or absence of light and aeration. They were only found in snails from long established balanced tanks and were absent from snails recently introduced into new balanced aquaria. Up to thirteen separate proteins could be seen on cellulose acetate strips. The pattern produced by four species was clearly similar, but in the other species the separations were less clear and did not resemble the common pattern.

These protein fractions are unlikely to affect the maintenance of blood equilibria since they appeared only sporadically in most species. There was no indication of the stimuli which induced their formation but since they occurred in blood from most of the species examined and formed a common pattern with four species which are widely separated taxonomically, they are probably

produced in response to a stimulus which is common to all snails and which might not be influenced by changes in the external environment. The proteins are unlikely to be of value for taxonomic studies primarily because they appear so infrequently; a more accurate method of estimating them would show, however, whether any of the fractions were species specific and how much variation occurred within a single species.

The mobility of haemoglobins from twelve species of snails was the same and was shown to be just over half that of baboon serum albumen. The mobility of haemocyanin from L. stagnalis was greater than that of the haemoglobins, and haemocyanin from O. hupensis travelled faster than baboon albumen. These rates were unaffected by changes in pH.

The similarity between the absorption spectra of haemoglobin from four snail species indicated that the prosthetic portions of the molecules were the same. This technique is therefore unlikely to yield further information for the characterisation of haemoglobin types. Similarly, since the mobilities of haemoglobins from twelve species were the same when examined by electrophoresis on cellulose acetate, the form of the whole molecule of the respiratory pigments is the same for

each species, any differences in their structure not being sufficient to affect the rate of movement under the conditions of these experiments. Electrophoresis will yield little further information, therefore, about the form of different haemoglobin molecules.

The similarity in the results obtained in the preceding experiments with different snail haemoglobins did not preclude the possibility of variations occurring in the amino acid composition of the globin portions of the molecules. The amino acids in four snail haemoglobins and one haemocyanin were therefore investigated. Tryptophane, which is present in mammalian haemoglobin, was not found in these pigments, but the other seventeen amino acids of the mammalian pigment were present. The relative amounts of eleven amino acids in three snail haemoglobins were estimated. Differences were found in the amounts of alanine, lysine and especially of aspartic and glutamic acids present in the three haemoglobins, showing that the amino acid composition of the pigments varied with the species.

The general structure of haemoglobin molecules from different species was the same and in consequence many of the properties are likely to be similar. The quantitative differences in the concentrations of some

globin amino acids may be sufficient, however, to produce differences in some functions of the molecules, for example the maintenance of ion and salt levels in the blood. Such factors may contribute to the successful development of schistosome parasites if the sporocysts require particular levels of some inorganic substances. Examinations are now required of the haemoglobins from more intermediate hosts, and a study of the properties and functions of the respiratory pigments should be made to determine whether differences in their amino acid composition produce significant effects on the environment of the parasite.

Qualitative estimations were made of the free amino acids which are readily available to different stages of the developing parasite. Twelve free amino acids were identified in the blood of three snail species; they were in very low concentration. Seventeen amino acids were found free in the digestive glands and ovotestes of these species, and also in hydrolysed proteins from the same organs. Tryptophane was never found in the blood or tissues and methionine, arginine, histidine and proline were also absent from the blood. Phenylalanine was identified in blood from laboratory reared P. corneus and L. stagnalis but not in blood from these species collected from natural habitats.

The schistosome intermediate host A. glabratus showed the same levels of amino acids in the blood, digestive gland and ovotestes as insusceptible snails. The secondary sporocysts develop in the digestive gland and ovotestes and, since the concentrations of amino acids in these organs were high in all the snails examined, there was no indication of factors which might limit development in this region to A. glabratus alone. The influence of the external environment on the amino acid content of the organs was also slight with only a single difference between snails from two habitats. This should be investigated for a larger number of snail species, particularly where a snail which acts as an intermediate host for schistosomes varies in its susceptibility to infection when taken from different habitats.

The free amino acids in the head, musculature and mantle of A. glabratus and of wild and cultivated P. corneus and L. stagnalis were identified. The cultivated L. stagnalis lacked arginine and histidine which were present in the wild snails. Only A. glabratus contained methionine, but this was not present in snails infected with S. mansoni. The concentrations of all amino acids were lower in these infected snails. The amino acids present in proteins from cercariae of S. mansoni were also identified. The seventeen amino

acids which occur in the snail host, including methionine, were detected in the parasite.

These results indicate that the developing schistosome obtains part of its amino acid requirements from the free amino acids of the snail. The amount of non-protein amino acids in the digestive glands and ovotestes of the snail is comparatively high but there was a marked reduction in amino acid concentration in infected snails. Free amino acids occur in low concentration in the anterior tissues of the snail but mature infections even depleted these tissues. This was particularly marked with methionine. This amino acid was detected in the anterior tissues of A. glabratus alone and it disappeared when the snails were infected. The methionine was therefore utilised by the parasite and, since this amino acid was not present in measurable amounts in insusceptible snails, the results indicate that the quantitative levels of one or more amino acids in the anterior tissues may determine, in part, whether a parasite will be able to develop in a particular snail.

This study has shown that the internal environment of a snail species could govern the susceptibility of the snail to infection by schistosomes,

and further investigations are proposed into the requirements of the parasites and the physiology of different snail species.

Future Work

1. The haemoglobins are similar in general structure and properties but the differences in amino acid composition may influence some of the properties of the molecules. Snail species which are more closely related taxonomically should be compared to determine whether their haemoglobins show species differences or whether there are haemoglobin types common to larger groups of snails.

2. Comparative studies of the properties of snail haemoglobins and haemocyanins should be initiated to determine whether these pigments affect the ability of a snail to become infected by schistosomes.

3. Blood proteins other than the pigment occurred only sporadically in all the snail species and there was no indication of the factors governing their appearance. Single snails should be examined over long periods of time, to establish the rate of occurrence of the protein fractions and the variability in concentrations.

The effect of emphasis or removal of environmental stimuli which might indicate the physiological factors affecting the proteins should also be examined over long periods of time.

4. The protein and non-protein amino acids of the digestive glands and blood of the snails examined were qualitatively similar but differences were obtained between the free amino acids of the other tissues. The high level of methionine in tissues of the intermediate host was particularly significant. Qualitative examinations of the free amino acids of other intermediate hosts of schistosomes are required with quantitative comparisons between the amino acids of these snails and those of non-susceptible snails from similar areas.

5. The results suggested that part at least of the amino acid requirements of the parasite were obtained from non-protein amino acids of the hosts. The bound amino acids of different strains and species of schistosomes should be determined, and the parasite should be examined at different stages of development for proteolytic enzymes, which would indicate whether it obtains any of its amino acids from proteins of the snail.

Appendix IProtein StainsA. Hanging strip paper electrophoresis1. Azocarmine B

Reference - Klinische Wochenschrift 29, (1951).

Azocarmine B 0.75 g.

Methanol 50 ml.

Glacial acetic acid 10 ml.

Water 50 ml.

Stain for 5 minutes

Wash with methanol plus 10% acetic acid
for 5 minutes.

Wash with 10% aqueous acetic acid until
the background is white.

Wash with water.

B. Horizontal paper electrophoresis1. Azocarmine B

As above

2. Light green

Reference - Block, Durrum and Zweig (1958).

Stain with 1% aqueous light green in 1%
acetic acid for 5-8 minutes.

3. 1% Nigrosine

Reference - M. Ortega (1957)

Stain with 1% water soluble nigrosine in 1% acetic acid for 6 minutes.

Wash with 1% acetic acid or tap water until the paper has a faint blue tinge.

C. Horizontal electrophoresis on cellulose acetate

1. Nigrosine

Reference - J. Kohn (1958)

Stain with 0.005% nigrosine in 2% acetic acid for 1-2 hours.

Wash several times with 2% acetic acid and blot dry.

Appendix IIBuffer Solutions for ElectrophoresisA. Hanging strip paper electrophoresis1. pH 5.2 Acetate buffer42.0 ml. 0.1N CH_3COOH 158.0 ml. 0.1N CH_3COONa 2. pH 8.6 Barbitone buffer 0.05M

1.84 g. barbital (diethyl barbituric acid)

10.3 g. soluble barbitone (sodium barbital)

Make up to 1 litre.

B. Horizontal paper electrophoresis1. pH 8.6 Barbitone buffer

As above

C. Horizontal electrophoresis on cellulose acetate1. pH 8.6 Barbitone buffer

As above

2. pH 11.6 Barbitone buffer

10 ml. 0.05M soluble barbitone

6 ml. 0.02M NaOH

3. pH 9.0-10.0 Borate buffer50 ml. 0.2M H_3BO_3 + 0.2M KCl

x ml. 0.2M NaOH

Dilute to 100 ml.

4. pH 9.32 (Theoretical pH 9.22)

Reference - Pearse (1953)

80 ml. 0.1M Glycine + 0.1M NaCl

20 ml. 0.1M NaOH

5. pH 11.2-12.0

Reference McFarren (1951)

50 ml. 0.067M Na_2HPO_4

x ml. 0.067M NaOH

(x = 16.5 - 50.0 ml.)

6. pH 11.6

10 ml. 0.01M soluble barbitone

15 ml. 0.004M NaOH

7. pH 11.63

10 ml. 0.05M soluble barbitone

2 ml. 0.05M NaOH

Appendix III

Buffer Solutions for Circular Paper Chromatography

Reference - McFarren, E.F. (1951)

pH 2.0 5ml. 0.067M KCl + 10.66 ml. 0.067M HCl

pH 8.4 50 ml. 0.067M H_3BO_3 and KCl + 8.55 ml.
0.067M NaOH

pH 12.0 50 ml. 0.067M Na_2HPO_4 + 50 ml. 0.067M NaOH

Appendix IV

Reclamation of Dowex-50 Resin

The resin column is washed with 2N.NaOH. This converts the resin to the Na^+ form. The column is washed with water and then with 4N.HCl until the effluent is free from sodium (flame test). The resin is washed again with water until all chloride ions are removed (as shown by silver nitrate) or until the pH of the effluent equals that of the water going in.

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